Studies on the compounds having suppressive effects on the activation of macrophages and mast cells(マクロファージ及び肥満細胞の活性化抑制作用を示す化合物に関する研究)

Studies on the Compounds having Suppressive Effects on the Activation of Macrophages and Mast Cells

マクロファージ及び肥満細胞の活性化抑制作用を示す化合物に関する研究）

Laboratory of Pathophysiological Biochemistry
Graduate School of Pharmaceutical Sciences
Tohoku University

Ban Hyun Seung

List of Abbreviations Used in This Thesis

AP-1: activator protein-1

COX: cyclooxygenase

cPGES: cytosolic prostaglandin E synthase

CS: calf serum

DMSO: dimethyl sulfoxide

DNP-HSA: dinitrophenol-conjugated human serum albumin

EDTA: ethylenediamine-*N,N,N',N'*-tetraacetic acid

EMEM: Eagle's minimal essential medium

EMSA: electrophoretic mobility shift assay

ERK: extracellular signal-regulated kinase

FBS: fetal bovine serum

$I_\kappa B$: inhibitory protein of NF-κB

IL: interleukin

JNK: c-Jun N-terminal kinase

LPS: lipopolysaccharide

LT: leukotriene

MAPK: mitogen-activated protein kinase

mPGES: membrane-associated prostaglandin E synthase

NF-κB: nuclear factor-κB

NO: nitric oxide

NSAID: non-steroidal anti-inflammatory drug

PBS: phosphate-buffered saline

PG: prostaglandin

PI3K: phosphatidylinositol 3-kinase

PKC: protein kinase C

PLA_2: phospholipase A_2

RA: rheumatoid arthritis

RT-PCR: reverse transcription-polymerase chain reaction

SDS: sodium dodecylsulphate

TNF: tumor necrosis factor

TPA: 12-*O*-tetradecanoylphorbol 13-acetate

Contents

Preface

Macrophages play a major role in host defense against infection and tumor development [1,2]. During inflammatory process, activation of macrophages by inflammatory stimuli causes release of various inflammatory cytokines and chemical mediators [3-6]. Lipopolysaccharide (LPS), one of the major component of outer membrane of gram-negative bacteria, stimulates immune cells, mainly macrophages, to release various mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-10, prostanoids, and nitric oxide (NO) [7]. Sepsis is a clinical syndrome frequently mediated by LPS, and in patients with sepsis, TNF-α produced by LPS plays a major role in the associated systemic toxicity [8]. TNF-α also induces the secretion of cytokines such as IL-1 and activates other immune cells including T cells [9]. Among the chemical mediators released from activated macrophages, the arachidonate metabolite prostaglandins (PGs) control pain, fever and inflammatory responses [10], and NO generated by NO synthase (NOS) induces tissue injury at the inflammatory site [11].

Mast cells also play a central role in the initiation of the immune response associated with allergic disorders and asthma [12]. Mast cells express FcεRI, a high-affinity receptor for IgE, on their surface [13]. Cross-linkage with the antigen of the IgE-FcεRI complex activates mast cells, and leads to degranulation and the production of cytokines and arachidonate metabolites [14]. Histamine stored in secretory granules is rapidly released in response to the antigen, and induces the contraction of smooth muscles, an increase in vascular permeability and the secretion of mucus [15]. The antigen also induces the production of arachidonic acid metabolites such as PGD_2, a chemoattractant for eosinophils [16], and leukotriene (LT) B_4, a chemoattractant for eosinophils and neutrophils [17], and peptide-LTs that increase vascular permeability [18]. Among the cytokines produced in response to the antigen, IL-4 and IL-13 induce production of IgE by B cells and induce Th2 cell development [19]. Therefore, modulation of macrophages and mast cell activation might be therapeutic targets for controlling inflammatory diseases.

The medicinal plants have been used for the treatment of various diseases since ancient

times. Therefore, medicinal plants have been recognized as a source of new therapeutic candidate compounds, and various pharmaceutical agents have been isolated from the medicinal plants. For example, salicylic acid was identified in 1839 as an active component in a number of plants known for their analgesic activity, and was first synthesized in 1853. It led to the development of aspirin, which is the most widely used synthetic drug today. Still now, a variety of studies has been carrying out to find lead compounds to develop more potent and less toxic drugs from the medicinal plants.

On the basis of this research current, I have studied to find lead compounds for anti-inflammatory drugs from natural resources and analyzed their mechanism of action. In cultures of macrophages and mast cells, I have studied effects of four kinds of natural products, furanocoumarins isolated from the roots of *Angelica dahurica* (Chapter I), lignans isolated from the roots of *Acanthopanax chiisanensis* (Chapter II), synthesized flavonoids 2'-hydroxychalcone derivatives (Chapter III), and stilbene resveratrol, a constituent of *Vitis vinifera* extract (Chapter IV), on the production of inflammatory chemical mediators and cytokines.

Chapter I. Inhibition of production of prostaglandin E_2 and tumor necrosis factor-α by furanocoumarins isolated from *Angelica dahurica*

I.1. Introduction

A arachidonate metabolite PGE_2 is produced by three enzymatic reactions; release of arachidonic acid from membrane phospholipids by phospholipase A_2 (PLA_2), conversion of arachidonic acid to PGH_2 by cyclooxygenase (COX), and isomerization of PGH_2 to PGE_2 by PGE synthase (PGES) [20]. It has been reported that there are two types of COX; COX-1 and COX-2, and three types of PGES; cytosolic PGES (cPGES), membrane-associated PGES (mPGES)-1 and mPGES-2 [21-23]. COX-1 and cPGES are constitutively expressed in most tissues [24], functionally coupled, and promote immediate PGE_2 production [25]. COX-2 and mPGES-1 are induced by various inflammatory stimuli [26-29], functionally coupled, and promote delayed PGE_2 production [30]. mPGES-2, a recently discovered third PGES, is constitutively expressed and functionally coupled with both COX-1 and COX-2 [31]. The inhibition of COX activity by non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin is at the center of current anti-inflammatory therapies. Recently, new classes of drugs targeting mPGES-1 are under development for the treatment of inflammatory diseases such as rheumatoid arthritis (RA) [32]. RA is a chronic disease characterized by systemic and local inflammation leading to joint destruction and functional impairment [33]. The proinflammatory cytokines including TNF-α and IL-1β play key roles in initiating RA, and these cytokines induce the production of PGE_2 which induces several pathologic features of RA such as pain and bone destruction [34,35]. Therefore, regulations of production of TNF-α and PGE_2 may be a therapeutic target in RA.

Angelica dahurica Benth. et Hook. (Umbelliferae) is one of the important medicinal plants in Korea and the roots of which have traditionally been used to reduce headache caused by cold, toothache, neuralgia and hemorrhage as Chinese medicines [36]. It is reported that the components from *Angelica dahurica* have various biological activities such as hepatoprotective activity against tacrine-induced cyotoxicity in Hep G2 cells [37], inhibitory activity against compound 40/80-induced histamine release in mouse peritoneal cavity [38], and

antimicrobial activity [39].

To clarify the anti-inflammatory effects of *Angelica dahurica*, I have examined effects of furanocoumarins isolated from the roots of *Angelica dahurica* and semi-synthesized compounds from the isolated furanocoumarins on LPS-induced production of PGE_2 and TNF-α in rat peritoneal macrophages and murine macrophage cell line RAW 264.7 cells.

I.2. Results

I.2.1. Effects of various fractions from the roots of *Angelica dahurica* on LPS-induced production of PGE_2

Incubation with LPS (0.1 μg/ml) for 8 h prominently increased PGE_2 production in rat peritoneal macrophages (Table 1). From the methanol extract of the roots of *Angelica dahurica*, *n*-hexane, methylene chloride, ethyl acetate and water fractions were obtained, and the effects of these fractions at 30 μg/ml on the LPS-induced PGE_2 production were examined.

Table 1. Effects of various fractions from the roots of *Angelica dahurica* on LPS-induced PGE_2 production.

Treatment	PGE_2 (ng/ml)
None	2.07 ± 0.15 ***
LPS (0.1 μg/ml)	8.40 ± 0.15 ###
LPS + methanol extract (30 μg/ml)	5.61 ± 0.21 ###
LPS + *n*-hexane fraction (30 μg/ml)	2.53 ± 0.11 ***
LPS + methylene chloride fraction (30 μg/ml)	1.96 ± 0.13 ***
LPS + ethyl acetate fraction (30 μg/ml)	6.54 ± 0.28 ###
LPS + water fraction (30 μg/ml)	8.40 ± 0.06 ###
LPS + indomethacin (0.1 μM)	1.89 ± 0.17 ***

Values are the means from four samples with the S.E.M. Statistical significance; ###P<0.001 vs. None; **P<0.01, ***P<0.001 vs. LPS control.

Except for the water fraction, all the fractions significantly inhibited the LPS-induced PGE_2 production (Table 1). Among these fractions, the methylene chloride fraction showed the most potent inhibitory activity, and followed by the *n*-hexane fraction. The inhibitory effect of the methylene chloride fraction at 30 μg/ml was almost the same as that of indomethacin at 0.1 μM (Table 1).

I.2.2. Effects of isolated furanocoumarins and semi-synthesized compounds on LPS-induced production of PGE_2

From the methylene chloride fraction and the *n*-hexane fraction, five furanocoumarins (Fig. 1) have been isolated and five semi-synthetic compounds from the isolated furanocoumarins (Fig. 2) have been prepared.

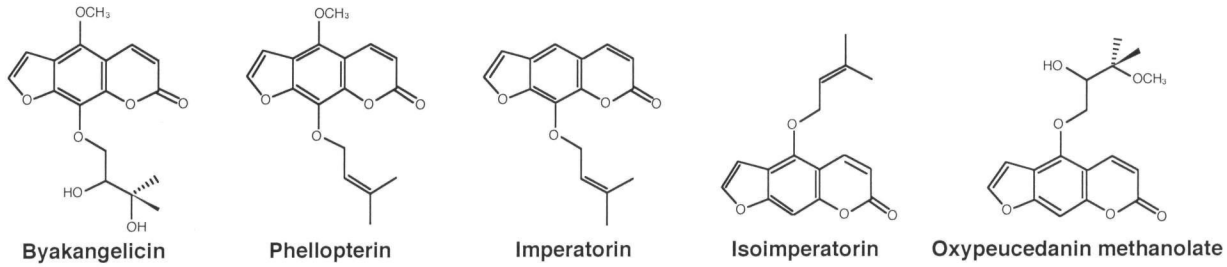


Figure 1. Chemical structures of furanocoumarins isolated from the roots of *Angelica dahurica*.

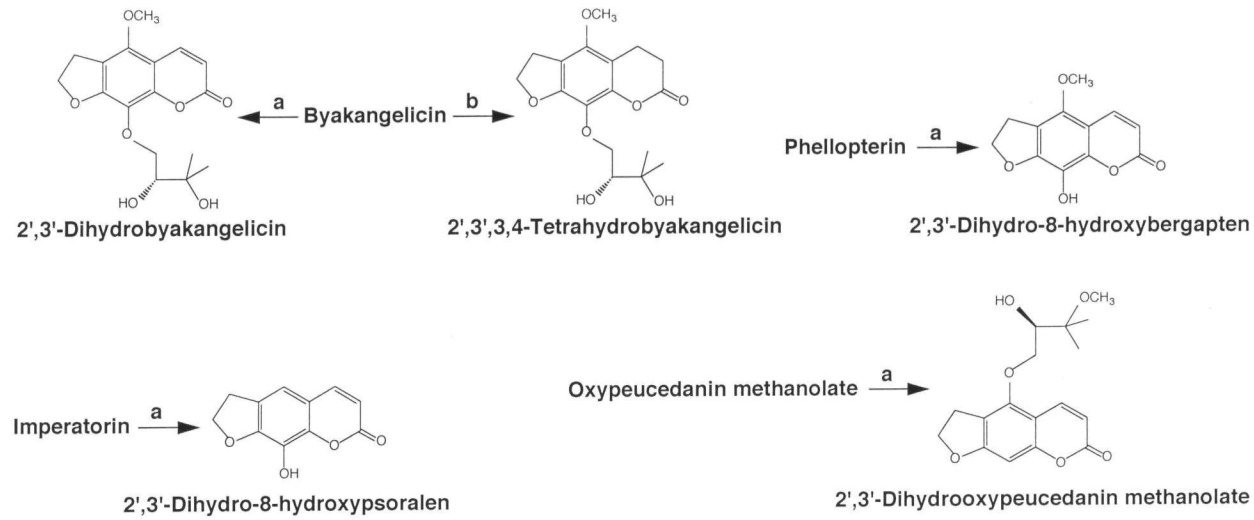


Figure 2. Preparation of semi-synthetic compounds from the isolated furanocoumarins.
a. H2, Pd/C 1 eq., 30 min, **b**. H2, Pd/C 2 eq., 1 h

Among the isolated furanocoumarins and semi-synthesized compounds, phellopterin, imperatorin and isoimperatorin inhibited the LPS-induced PGE_2 production more effectively than other compounds at 30 μM. The inhibitory effects of these compounds at 30 μM were almost the same as that of indomethacin at 0.1 μM (Fig. 3).

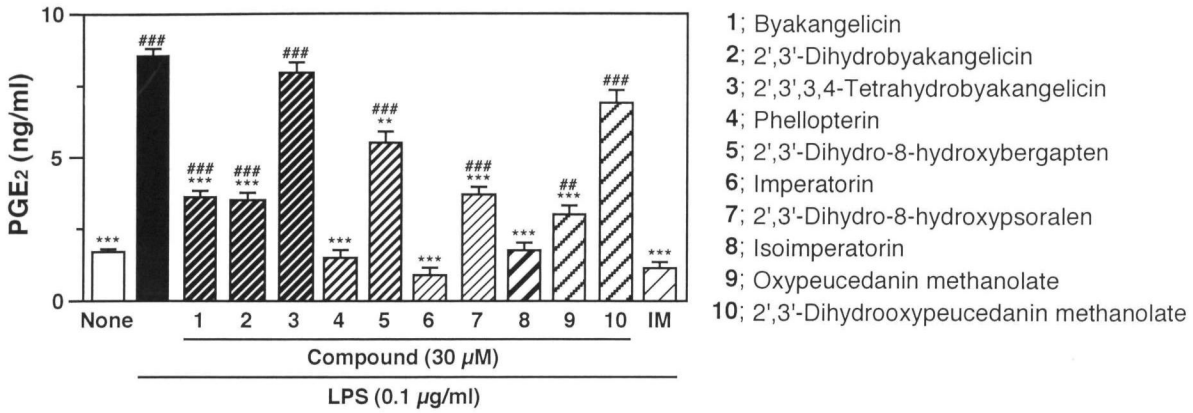


Figure 3. Effects of the isolated and semi-synthesized furanocoumarins on LPS-induced PGE_2 production. Rat peritoneal macrophages (7.5×10^5 cells) were incubated for 8 h in 0.5 ml of medium containing each compound (30 μM) or indomethacin (IM, 0.1 μM) in the presence of LPS (0.1 μg/ml). The concentration of PGE_2 in the conditioned medium was measured by radioimmunoassay. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. None; $^{**}P<0.01$, $^{***}P<0.001$ vs. LPS control.

I.2.3. Effects of imperatorin on the protein levels of COX-1, COX-2, cPGES and mPGES-1

To clarify the mechanism of action for the inhibition of PGE_2 production by furanocoumarins, the effects of imperatorin on the LPS-induced increase in the levels of COX-2 and mPGES-1 were examined. As shown in Fig. 4A, imperatorin at 3 to 30 μM inhibited the LPS-induced PGE_2 production at 8 h in a concentration-dependent manner, and at 3 μM, PGE_2 production was suppressed to 49% that of LPS control. Treatment with LPS (0.1 μg/ml) for 8 h induced the expression of COX-2 and mPGES-1 (Fig. 4B). In the presence of dexamethasone (0.1 μM), both the LPS-induced increases in the levels of COX-2 and mPGES-1 were suppressed. Imperatorin at 3 to 30 μM also suppressed the LPS-induced expression of COX-2 and mPGES-1 in a concentration-dependent manner (Fig. 4B). The levels of COX-1 and cPGES were not affected by treatment with LPS alone, or with LPS and imperatorin (Fig. 4B). These findings suggest that the inhibitory effect of imperatorin on the LPS-induced PGE_2 production is due to the inhibition of the expression of both COX-2 and mPGES-1.

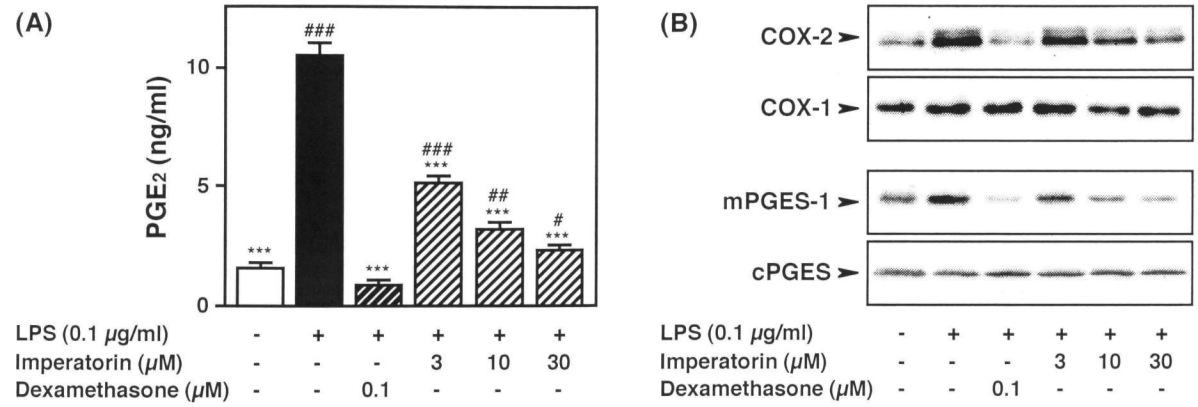


Figure 4. Effects of imperatorin on LPS-induced expressions of COX-1, COX-2, cPGES and mPGES-1. Rat peritoneal macrophages (1.5 x 10^6 cells/ml) were incubated for 8 h in 0.5 ml (A) or 2 ml (B) of medium containing the indicated concentration of imperatorin or dexamethasone (0.1 μM) in the presence of LPS (0.1 μg/ml). (A) The concentration of PGE_2 in the conditioned medium was measured by radioimmunoassay. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. Non-stimulated control; $^{***}P<0.001$ vs. LPS control. (B) The protein levels of each COX and PGES were determined by immunoblotting.

I.2.4. Effects of imperatorin on LPS-induced increases in the level of TNF-α mRNA and protein

The treatment of RAW 264.7 cells with LPS (0.1 μg/ml) increased the level of TNF-α mRNA at 4 h (Fig. 5A) and TNF-α protein at 6 h (Fig. 5B). Imperatorin at 3 to 30 μM suppressed the LPS-induced increase in the level of TNF-α mRNA and protein in a concentration-dependent manner (Fig. 5A and B). These findings suggest that imperatorin inhibits the LPS-induced TNF-α production by suppressing the expression of TNF-α mRNA.

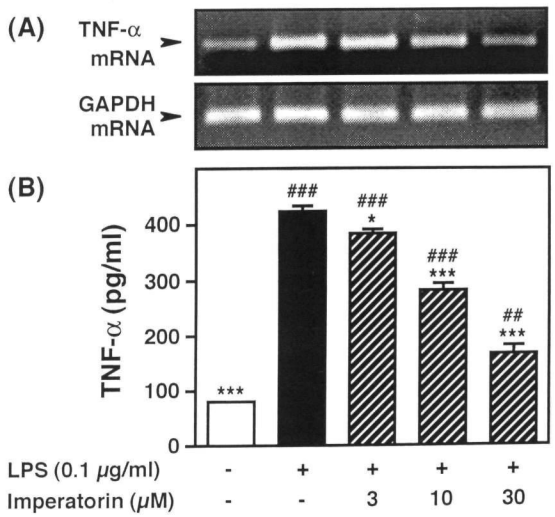


Figure 5. Effects of imperatorin on LPS-induced expression of TNF-α. RAW 264.7 cells (5 x 10^5 cells/ml) were incubated for 4 h (A) or 6 h (B) in 1 ml (A) or 0.5 ml (B) of medium containing LPS (0.1 μg/ml) and the indicated concentration of imperatorin. (A) The levels of mRNA for TNF-α and GAPDH were determined by RT-PCR. (B) The concentration of TNF-α in the conditioned medium was measured by ELISA. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. Non-stimulated control; $^{*}P<0.05$, $^{***}P<0.001$ vs. LPS control.

I.2.5. Effects of inhibitors of MAPKs and NF-κB on LPS-induced production of TNF-α

The involvement of MAPKs and NF-κB in LPS-induced expression of TNF-α was analyzed. As shown in Fig. 6, in the presence of U0126 (MEK inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor) or Bay II-7082 (NF-κB inhibitor), the LPS-induced production of TNF-α was significantly suppressed. These findings suggest that the activations of MAPKs and NF-κB up-regulate expression of TNF-α in LPS-stimulated macrophages.

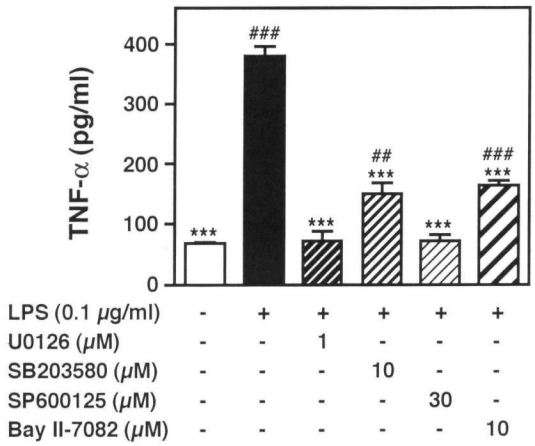


Figure 6. Effects of inhibitors of MAPKs and NF-κB on LPS-induced expression of TNF-α. RAW 264.7 cells (5 x 10^5 cells/ml) were incubated for 6 h in 0.5 ml of medium containing LPS (0.1 μg/ml) and the indicated concentration of the MEK inhibitor U0126, the p38 MAPK inhibitor SB203580, the JNK inhibitor SP600125 or the NF-κB inhibitor Bay II-7082. The concentration of TNF-α in the conditioned medium was measured by ELISA. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. Non-stimulated control; $^{***}P<0.001$ vs. LPS control.

I.2.6. Effects of imperatorin on LPS-induced phosphorylation of MAPKs

The treatment with LPS (0.1 μg/ml) for 30 min induced phosphorylation of ERK, p38 MAPK and JNK (Fig. 7). At 3 to 30 μM, imperatorin did not affect the LPS-induced phosphorylation of each kinase (Fig. 7). These findings indicate that the inhibition by imperatorin of the LPS-induced TNF-α production is not mediated through suppression of MAPKs activation.

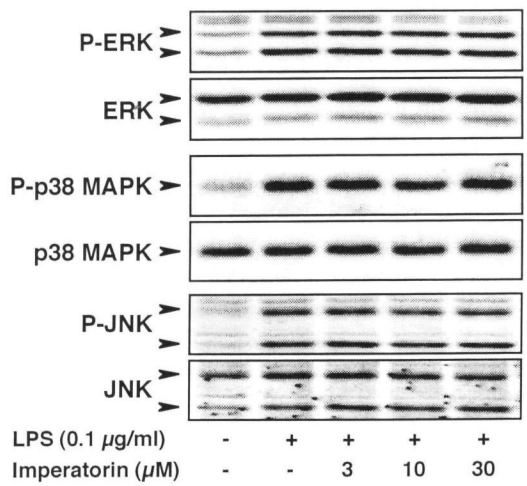


Figure 7. Effects of imperatorin on LPS-induced phosphorylation of MAPKs. RAW 264.7 cells (5 x 10^5 cells/ml) were incubated for 20 min in 2 ml of medium containing LPS (0.1 μg/ml) and the indicated concentration of imperatorin. The protein levels of phosphorylated (P)-ERK, ERK, phosphorylated (P)-p38 MAPK, p38 MAPK, phosphorylated (P)-JNK, and JNK were determined by immunoblotting.

I.2.7. Effects of imperatorin on LPS-induced activation of NF-κB

The treatment with LPS (0.1 μg/ml) increased NF-κB-mediated luciferase activity at 6 h (Fig. 8A) and binding of NF-κB to its specific oligonucleotide probe at 1 h (Fig. 8B). Imperatorin at 3 to 30 μM decreased the LPS-induced luciferase activity and DNA binding of NF-κB (Fig. 8A and B). Bay II-7082 at 10 μM also suppressed the activation of NF-κB (Fig. 8A and B). These findings suggest that imperatorin inhibits the LPS-induced production of TNF-α by suppressing the activation of NF-κB.

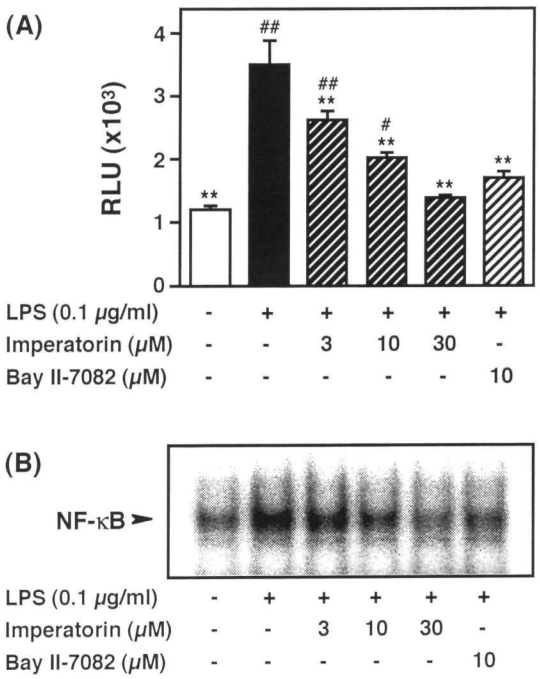


Figure 8. Effects of imperatorin on LPS-induced activation of NF-κB. (A) RAW264.7 cells (6 x 10^6 cells) were transfected with 30 μg of pNF-kB-Luc, and incubated for 12 h in 24-well cell culture dishes (2.5 x 10^5 cells/0.5 ml/well). The cells were further incubated for 6 h in 0.5 ml of medium containing LPS (0.1 μg/ml) and the indicated concentrations of imperatorin or Bay II-7082, and luciferase activity was measured as relative luciferase unit (RLU). Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. Non-stimulated control; $^{**}P<0.01$ vs. LPS control. (B) RAW 264.7 cells (5 x 10^5 cells/ml) were incubated for 1 h in 4 ml of medium containing LPS (0.1 μg/ml) and the indicated concentration of imperatorin or Bay II-7082. The nuclear protein was extracted, and the levels of NF-κB bound to the DNA probe was determined by EMSA.

I.2.8. Effects of imperatorin on LPS-induced degradation of IκB-α

The treatment with LPS (0.1 μg/ml) for 30 min induced degradation of IκB-α (Fig. 9). In the presence of imperatorin at which concentrations NF-κB activation was inhibited, the LPS-induced degradation of IκB-α was not affected (Fig. 9). Bay II-7082 at 10 μM potently suppressed the degradation (Fig. 9). These findings indicate that inhibition by imperatorin of NF-κB activation is not mediated through suppression of the LPS-induced IκB-α degradation.

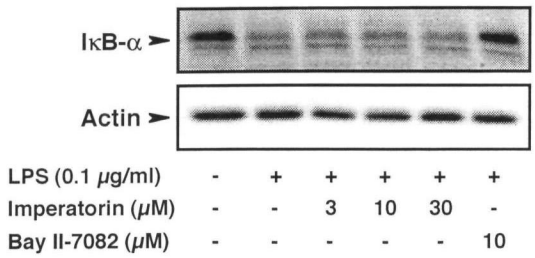


Figure 9. Effects of imperatorin on LPS-induced degradation of IκB-α. RAW 264.7 cells (5 x 10^5 cells/ml) were incubated for 30 min in 2 ml of medium containing LPS (0.1 μg/ml) and the indicated concentration of imperatorin. The protein levels of IκB-α and actin were determined by immunoblotting.

I.2.9. Effects of imperatorin on binding of NF-κB to DNA *in vitro*

The nuclear proteins extracted from LPS-treated cells were incubated with imperatorin or Bay II-7082 *in vitro*. As shown in Fig. 10, imperatorin at 30 and 100 μM inhibited the increase in the binding of NF-κB to DNA. However, Bay II-7082 at 10 μM did not affect it. These findings suggest that imperatorin inhibits the activation of NF-κB by suppressing the binding of NF-κB to DNA.

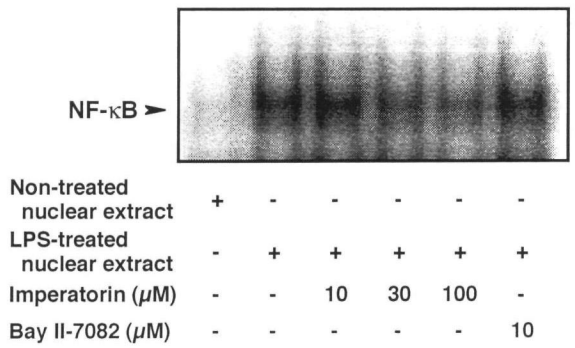


Figure 10. Effects of imperatorin on binding of NF-κB to DNA *in vitro*. RAW 264.7 cells (5 x 10^5 cells/ml) were incubated for 1 h in 4 ml of medium containing LPS (0.1 μg/ml). The nuclear protein was extracted, and 4 μg of protein was incubated for 30 min at 37°C with the indicated concentration of imperatorin or Bay II-7082. The levels of NF-κB bound to the DNA probe was determined by EMSA.

I.2.10. Effects of dithiothreitol on imperatorin-induced inhibition of NF-κB activation

Because α,β-unsaturated carbonyl compounds react directly with cysteine sulfhydry group of NF-κB [40], influence of dithiothreitol (DTT) having two sulfhydryl groups on imperatorin-induced inhibition of NF-κB activation was examined. As shown in Fig. 11, the LPS-induced binding of NF-κB to DNA was inhibited by imperatorin at 30 μM. In the presence of DTT, the inhibitory effect of imperatorin on the LPS-induced DNA binding of NF-κB was reduced (Fig. 11). These findings suggest that imperatorin reacts with NF-κB and inhibits its binding to DNA.

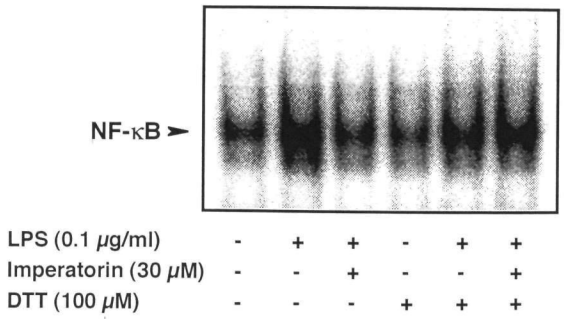


Figure 11. Effects of dithiothreitol on imperatorin-induced inhibition of NF-κB activation. RAW 264.7 cells (5 x 10^5 cells/ml) were incubated for 1 h in 4 ml of medium containing LPS (0.1 μg/ml) and imperatorin (30 μM) in the presence or absence of dithiothireitol (DTT, 100 μM). The nuclear protein was extracted, and the levels of NF-κB bound to the DNA probe was determined by EMSA.

I.3. Discussion

Among the isolated furanocoumarins and semi-synthesized compounds, phellopterin, imperatorin and isoimperatorin showed potent inhibitory activity for the LPS-induced PGE_2 production (Fig. 3), and the inhibition by imperatorin is mediated through suppression of induction of COX-2 and mPGES-1 (Fig. 4). Furthermore, the inhibitory activity of the semi-synthesized compounds did not exceed over that of the isolated furanocoumarins. For example, phellopterin showed more potent inhibitory activity than 2',3'-dihydro-8-hydroxybergapten, a partially reduced form of phellopterin (Fig. 3). These findings suggest that double bonds in furan ring and pyrano-2-one ring, and R1 and R2 substitutions are important for the expression of the inhibitory activity on the LPS-induced PGE_2 production.

Activation of NF-κB induces proinflammatory cytokines such as IL-1β and TNF-α, which activate the NF-κB [41]. Thus, the positive auto-regulation may increase the inflammatory response and prolong the duration of chronic inflammation. NF-κB also stimulates the expression of enzymes whose products contribute to the pathogenesis of the inflammatory process, including inducible NOS (iNOS) and COX-2 [41]. As shown in Fig. 4, imperatorin inhibited the LPS-induced COX-2 expression at which concentrations the activation NF-κB was inhibited (Fig. 8). It is also reported that imperatorin inhibits LPS-induced NO production through suppression of iNOS expression in RAW 264.7 [42]. Therefore, it is suggested that the inhibition by imperatorin of production of PGE_2, TNF-α and NO from the activated macrophages is mediated through suppression of the activation of NF-κB. In case of mPGES-1, it is reported that GC boxes are critical for its promoter activity, where a

transcription factor Egr-1 binds and triggers the transcription [43], and NF-κB also partially participates in the expression of mPGES-1 [44]. In addition to NF-κB, MAPKs are another major signaling molecules for the induction of the proinflammatory proteins including COX-2, iNOS and TNF-α in LPS-stimulated macrophages [7]. Activated MAPKs induce transactivation of transcription factors such as cAMP response element (CRE), activator protein (AP)-1, and CCAAT/enhancer binding protein (CEBP)-β that also contribute to expression of the proinflammatory proteins [45]. Because imperatorin did not affect the LPS-induced phosphorylation of MAPKs (Fig. 7), it is suggested that the inhibitory effects of imperatorin on the expression of COX-2 and TNF-α are mainly mediated by suppression of NF-κB activation.

The imperatorin-mediated inhibition of NF-κB activation was reversed by DTT (Fig. 11), suggesting that imperatorin modifies a sulfhydryl group in NF-κB. It is reported that cysteine 38 and 62 of NF-κB bind to DNA by forming hydrogen bond with sugar/phosphate backbone of the κB-DNA motif [46], and several studies have been reported that α,β-unsaturated carbonyl compound react directly with NF-κB [40,47,48]. 4-Hydroxy-2-nonenal, an α,β-unsaturated carbonyl compounds directly inhibited DNA binding of NF-κB and reduced LPS-induced production of NO in vascular smooth muscle cells [47]. Moreover, a sesquiterpene lactone helenalin [40] and a triterpenoid saponin avicins [48] also inhibited activation of NF-κB with a similar action mechanism of imperatorin (Fig. 10).

Besides the inhibition of NF-κB activation, it is reported that the α,β-unsaturated carbonyl compounds have a chemopreventive activity by inducing phase II enzymes including glutathione *S*-transferase, NAD(P)H:quinone reductase and epoxide hydrolase [49]. A transcription factor Nrf2 that induces phase II enzymes normally localized in cytosol binds to a chaperone, Keap1 [49]. By the reaction of α,β-unsaturated carbonyl compounds with cysteine in Keap1, Nrf2 released from Keap1 translocates into nucleus and binds to promoter region of phage II enzymes [50]. Avicins and other compounds having α,β-unsaturated carbonyl group induced the activation of Nrf2 and the inhibition of NF-κB by the reaction with cysteine [51]. Thus, these compounds including imperatorin may be potential therapeutic agents having various biological activities including anti-inflammatory and chemopreventive activities.

In conclusion, imperatorin isolated from the roots of *Angelica dahurica* showed the potent inhibitory activity on the LPS-induced PGE_2 production by suppressing the induction of COX-2 and mPGES-1 in rat peritoneal macrophages. Furthermore, imperatorin reduced the LPS-induced TNF-α production by suppressing the activation of NF-κB in RAW 264.7 cells. Therefore, it is suggested that imperatorin might be a lead compound for anti-inflammatory drug having a suppressive activity against the activation of macrophages.

Chapter II. Inhibition of prostaglandin E_2 production by lignans isolated from *Acanthopanax chiisanensis* in rat peritoneal macrophages

II.1. Introduction

NSAIDs inhibit synthesis of prostaglandin by inhibiting activity of COX, and have been widely used in the treatment of inflammatory diseases. Most NSAIDs exhibit analgesic and antipyretic activities, and some of NSAIDs have anti-thrombogenic activity [52]. Furthermore, several studies have reported that the relative risk of colorectal cancer was decreased by 40-50% in continuous users of aspirin or other NSAIDs, suggesting that these drugs may have chemopreventive activity [53]. However, NSAIDs have side-effects such as gastrointestinal injury [54]. Therefore, more potent and less side-effect anti-inflammatory drugs are still under development for the treatment of inflammatory diseases.

The roots and stem barks of *Acanthopanax* species, medicinal plants having ginseng-like activities, have traditionally been used for the treatment of rheumatoid arthritis and diabetes mellitus [55,56]. Among *Acanthopanax* species, *Acanthopanax chiisanenesis* Nakai (Araliaceae) is one of the most abundant indigenous species distributed in Mt. Jiri in Korea, however, its mechanism of action for anti-inflammatory activity has not been analyzed.

In this study, to clarify the anti-inflammatory activities of *Acanthopanax chiisanensis*, effects of five lignan compounds (*l*-sesamin, helioxanthin, savinin, taiwanin C and *cis*-dibenzylbutyrolactone) from the roots of *Acanthopanax chiisanensis* on PGE_2 production in rat peritoneal macrophages were examined.

II.2. Results

II.2.1. Effects of various fractions from the roots of *Acanthopanax chiisanensis* on TPA-induced PGE_2 production

The incubation of rat peritoneal macrophages for 8 h in the presence of TPA (30 nM) induced a prominent increase in PGE_2 production (Table 2). Among four fractions from the methanol extract of the roots of *Acanthopanax chiisanensis*, the chloroform fraction and ethyl

Table 2. Effects of various fractions from the roots of *Acanthopanax chiisanensis* on TPA-induced PGE_2 production.

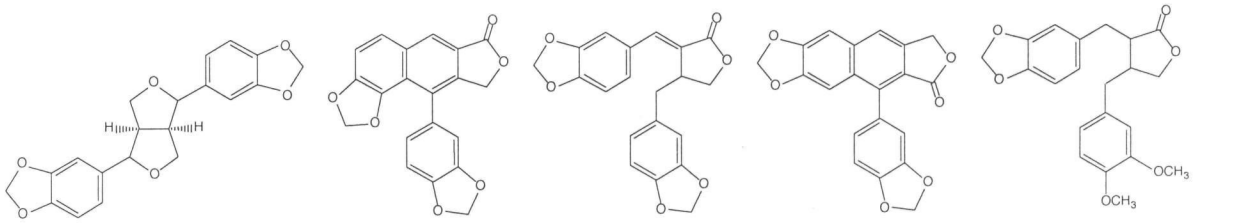
Treatment	PGE_2 (ng/ml)
None	2.10 ± 0.04 ***
TPA (30 nM)	8.48 ± 0.57 ###
TPA + *n*-hexane fraction (100 μg/ml)	8.28 ± 0.59 ###
TPA + chloroform fraction (100 μg/ml)	2.11 ± 0.12 ***
TPA + ethyl acetate fraction (100 μg/ml)	3.58 ± 0.33 ***
TPA + *n*-butanol (100 μg/ml)	8.63 ± 0.75 ###

Values are the means from four samples with the S.E.M.
Statistical significance; $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ vs. None;
$^{***}P<0.001$ vs. TPA control.

acetate fraction at 100 μg/ml showed a potent inhibitory activity against the LPS-induced PGE_2 production (Table 2). However, the *n*-hexane fraction and *n*-butanol fraction at 100 μg/ml had no effect (Table 2).

II.2.2. Effects of lignans on TPA-induced PGE_2 production

From the chloroform fraction, five lignans have been isolated (Fig. 12).



l-Sesamin Helioxanthin Savinin Taiwanin C *cis*-Dibenzylbutyrolactone

Figure 12. Chemical structures of five lignans isolated from the root of *Acanthopanax chiisanensis*.

Among the five lignans, taiwanin C showed the most potent inhibitory effect on the TPA-induced PGE_2 production with an IC_{50} value of 0.12 μM. At 30 μM, helioxanthin, *cis*-dibenzylbutyrolactone and savinin inhibited the TPA-induced PGE_2 production by 46.2%, 38.9%, and 26.2%, respectively, while *l*-sesamin showed no significant effect (Fig. 13). Under this condition, indomethacin as a positive control inhibited the TPA-induced PGE_2 production with an IC_{50} value of 0.01 μM (Fig. 13).

TPA (30 nM) stimulated PGE_2 production in a time-dependent manner from 4 to 24 h, and taiwanin C at 1 μM showed almost complete inhibition until 24 h as indomethacin at 0.1 μM did (Fig. 14).

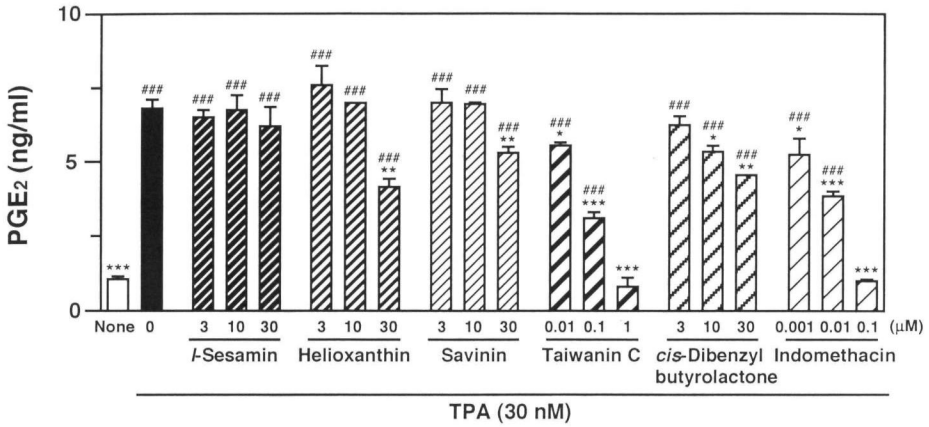


Figure 13. Effects of various concentrations of lignans on TPA-induced PGE_2 production in rat peritoneal macrophages. Rat peritoneal macrophages (7.5 x 10^5 cells) were incubated for 8 h in 0.5 ml of medium containing TPA (30 nM) and the indicated concentration of each lignan. The concentration of PGE_2 in the conditioned medium was measured by radioimmunoassay. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance; ###P<0.001 vs. None; *P<0.05, **P<0.01, ***P<0.001 vs. TPA control.

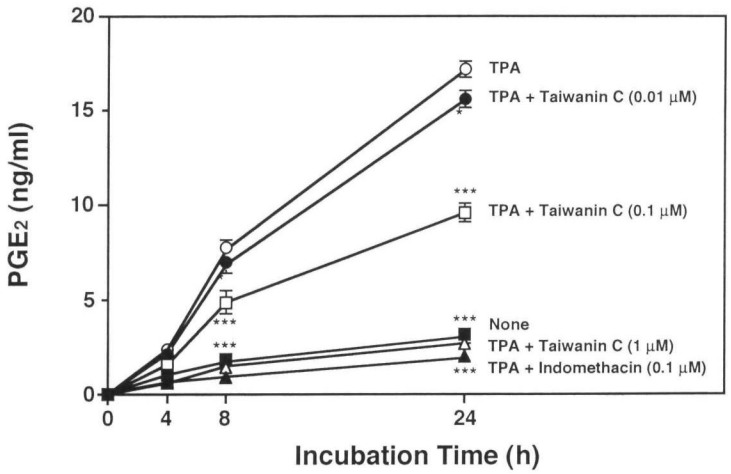


Figure 14. Time course of the effect of taiwanin C on TPA-induced PGE_2 production. Rat peritoneal macrophages (7.5 x 10^5 cells) were incubated for the periods indicated in 0.5 ml of medium containing TPA (30 nM) and the indicated concentration of taiwanin C or indomethacin. The concentration of PGE_2 in the conditioned medium was measured by radioimmunoassay. Values are the means from four samples with the S.E.M. shown by vertical bars. The symbols without the S.E.M. bars mean that the S.E.M. is within each symbol. Statistical significance; *P<0.05; TPA vs. TPA + Taiwanin C (0.01 μM) at 4, 8 and 24 h. ***P<0.001; TPA vs. TPA + Taiwanin C (0.1 μM), TPA + Taiwanin C (1 μM), TPA + Indomethacin (0.1 μM) and None at 4, 8 and 24 h.

II.2.3. Effects of taiwanin C on COX-1- and COX-2-dependent PGE_2 production

In the dexamethasone (10 μM)-pretreated macrophages, PGE_2 production at 4 h in the presence of dexamethasone (10 μM) was prominently increased by the addition of arachidonic acid (10 μM) (COX-1-dependent PGE_2 production), and indomethacin inhibited the production with an IC_{50} value of 0.33 μM (Fig. 15A). However, the COX-2 specific

inhibitor NS-398 showed no inhibitory activity at 0.01 to 1 μM. In the aspirin (100 μM)-pretreated macrophages, PGE_2 production increased markedly on the addition of TPA (30 nM) at 4 h (COX-2-dependent PGE_2 production), and indomethacin and NS-398 inhibited the production with an IC_{50} value of 0.01 μM and 0.005 μM, respectively (Fig. 15B). At 1 μM, taiwanin C inhibited the COX-1-dependent PGE_2 production by 46.9% (Fig. 15A). The COX-2-dependent PGE_2 production was also inhibited by taiwanin C, with an IC_{50} value of 0.23 μM (Fig. 15B).

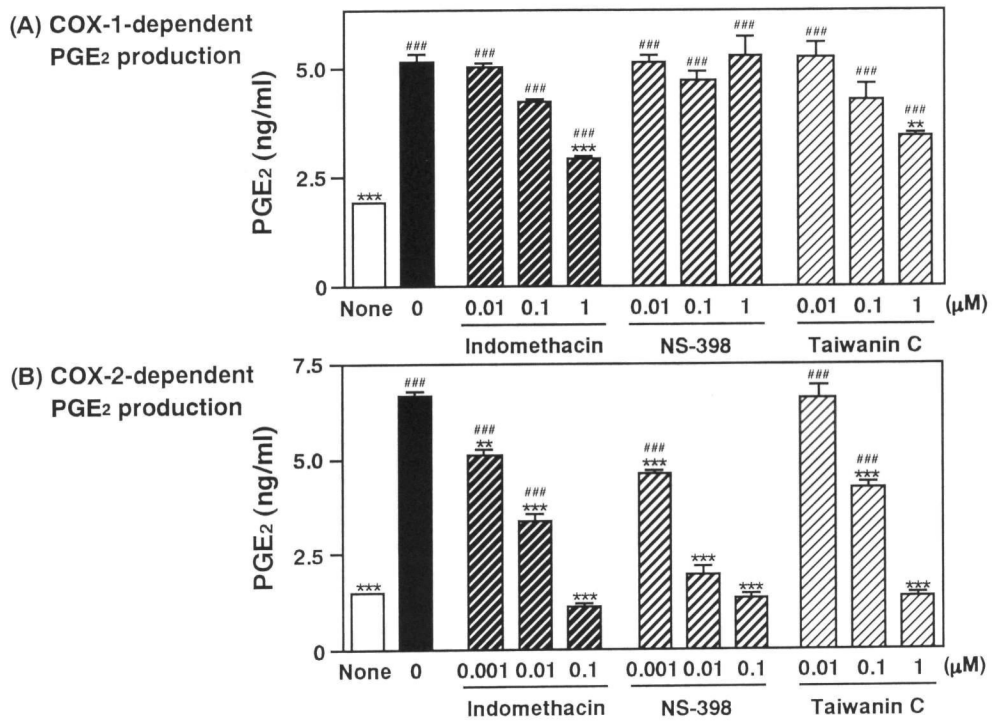


Figure 15. Effects of taiwanin C on COX-1-dependent PGE_2 production and COX-2-dependent PGE_2 production. (A) Rat peritoneal macrophages (7.5 x 10^5 cells) were preincubated for 12 h in 0.5 ml of medium containing dexamethasone (10 μM). After three washes, the cells were further incubated for 4 h in 0.5 ml of medium containing dexamethasone (10 μM) and the indicated concentration of indomethacin, NS-398 or taiwanin C in the presence of arachidonic acid (10 μM), and the concentration of PGE_2 in the conditioned medium was measured by radioimmunoassay (COX-1-dependent PGE_2 production). (B) Another set of macrophages (7.5 x 10^5 cells) were preincubated for 12 h in 0.5 ml of medium containing aspirin (100 μM). After three washes, the cells were further incubated for 4 h in 0.5 ml of medium containing the indicated concentration of indomethacin, NS-398 or taiwanin C in the presence of TPA (30 nM), and the concentration of PGE_2 in the conditioned medium was measured by radioimmunoassay (COX-2-dependent PGE_2 production). Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance; ###P<0.001 vs. None; **P<0.01, ***P <0.001 vs. indicated control.

II.2.4. Effects of taiwanin C on TPA-induced release of radioactivity from [^{3}H]arachidonic acid-labeled macrophages

TPA (30 nM) stimulated the release of radioactivity from [^{3}H]arachidonic acid-labeled

macrophages into the medium at 1 to 4 h, and taiwanin C at 0.01 to 1 μM showed no effect on the TPA-induced release of radioactivity (Fig. 16). These findings suggest that the inhibition of PGE_2 production by taiwanin C is not due to the inhibition of phospholipase A_2.

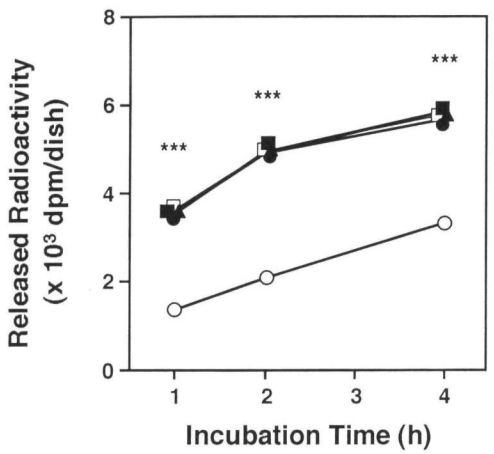


Figure 16. Effects of taiwanin C on the release of radioactivity from [^{3}H]arachidonic acid-labeled rat peritoneal macrophages. Rat peritoneal macrophages (7.5 x 10^5 cells) were incubated for 20 h in medium containing 3.7 kBq of [^{3}H]arachidonic acid. After three washes, the cells were incubated for the periods indicated in 0.5 ml of medium in the presence of TPA (30 nM) and various concentrations of taiwanin C (●; 0 μM, □; 0.01 μM, ■; 0.1 μM, and △; 1 μM). The amount of radioactivity released into the conditioned medium was determined. The radioactivity released from non-stimulated macrophages is shown by open circles. Values are the means from four samples. The S.E.M. of each value is too small to depict. Statistical significance; $^{***}P<0.001$ vs. non-stimulated control.

II.2.5. Effects of taiwanin C on the protein levels of COX-1 and COX-2

After treatment with TPA (30 nM) for 6 h, the COX-2 protein level was increased, while the COX-1 protein level did not change (Fig. 17). Treatment with taiwanin C at 0.01 to 1 μM in the presence of TPA (30 nM) did not affect the protein levels of COX-1 or COX-2 (Fig. 17). These findings indicate that the inhibition of PGE_2 production by taiwanin C is not due to the inhibition of the expression of COX-2 protein.

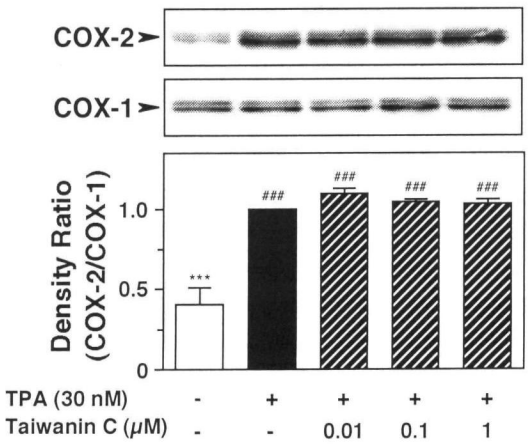


Figure 17. Effects of taiwanin C on the protein levels of COX-1 and COX-2 in TPA-stimulated rat peritoneal macrophages. Rat peritoneal macrophages (3 x 10^6 cells) were incubated for 6 h in 2 ml of medium containing TPA (30 nM) and the indicated concentration of taiwanin C. The protein levels of COX-1 and COX-2 were determined by immunoblotting. The density ratios of COX-2 protein to COX-1 protein were calculated, and the value of the density ratio in the TPA-treated control group is set to 1.0. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance; $^{###}P<0.001$ vs. Non-stimulated control; $^{***}P<0.001$ vs. TPA control.

II.2.6. Effects of taiwanin C on the activities of isolated COX-1 and COX-2

Indomethacin, a COX-1/COX-2 non-selective inhibitor, inhibited the activity of COX-1 and COX-2 in a concentration-dependent manner at 0.01 to 1 μM; the IC_{50} values for COX-1 and COX-2 being 0.09 μM and 0.16 μM, respectively. Taiwanin C also inhibited the activity of COX-1 (Fig. 18A) and COX-2 (Fig. 18B); the IC_{50} values for COX-1 and COX-2 were 1.06 μM and 9.31 μM, respectively. NS-398, a COX-2 specific inhibitor, inhibited COX-2 activity in a concentration-dependent manner at 0.01 to 1 μM (IC_{50} value; 0.11 μM), but showed no inhibitory effect on COX-1 at such concentrations. These findings suggest that the inhibition of PGE_2 production by taiwanin C is due to the direct inhibition of the activities of both the COX-1 and COX-2 enzymes.

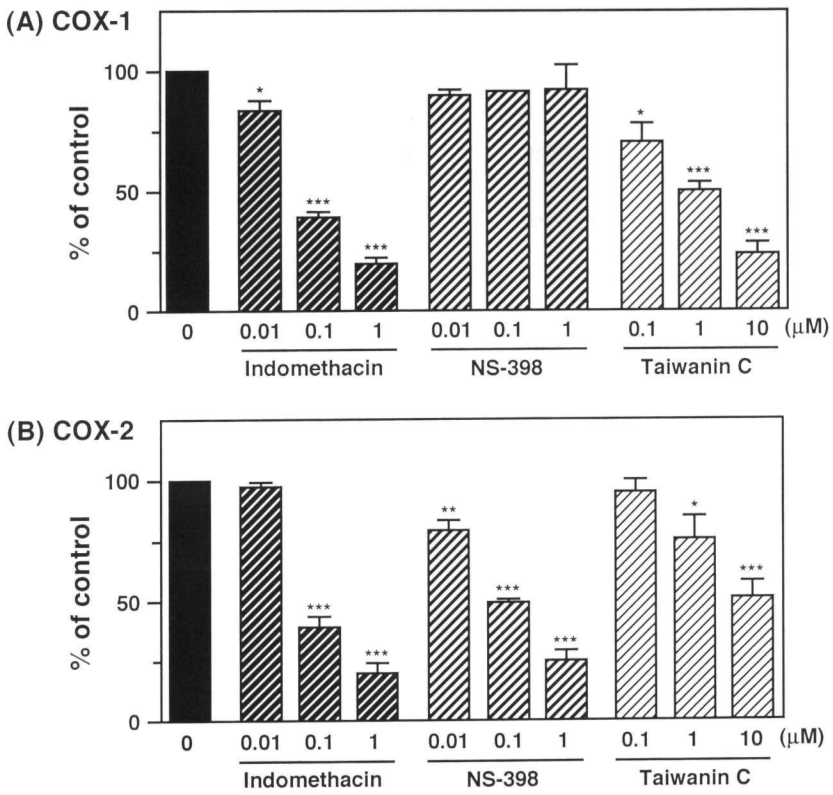


Figure 18. Effects of taiwanin C on the activity of isolated COX-1 and COX-2. One unit of COX-1 (isolated from sheep seminal vesicles) (A) and one unit of COX-2 (isolated from sheep seminal placenta) (B) dissolved in 210 μl of 100 mM Tris-HCl (pH 7.4) containing 10 mM EDTA, 1 mM reduced glutathione, 1 μM hematin and 0.5 mM phenol were incubated for 3 min at 37°C in the presence of the indicated concentration of taiwanin C, indomethacin, or NS-398. Arachidonic acid (0.1 μM) was then added, and the incubation continued at 37°C for another 3 min. The concentration of PGE2 in the reaction mixture was then measured by radioimmunoassay, and the concentration in the control group is set to 100%. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. corresponding control.

II.2.7. Effects of taiwanin C on thapsigargin- and staurosporine-induced PGE_2 production

To show that the inhibitory activity of taiwanin C is not specific to the TPA-induced PGE_2 production, effects of taiwanin C on PGE_2 production stimulated by other drugs including thapsigargin, an endomembrane Ca^{2+}-ATPase inhibitor, and staurosporine, a non-specific protein kinase inhibitor, were examined. In the presence of thapsigargin (30 nM) or staurosporine (30 nM), PGE_2 production was prominently increased at 8 h (Fig. 19). On treatment with taiwanin C (1 μM), the thapsigargin- and staurosporine-induced PGE_2 production was strongly inhibited as in the case of the TPA-induced PGE_2 production (Fig. 19).

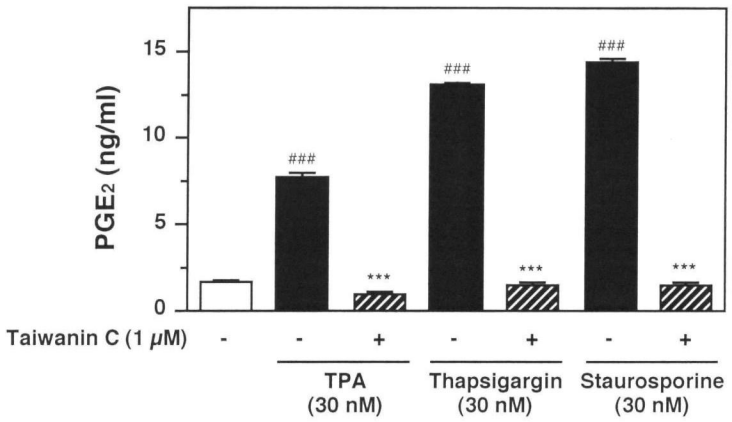


Figure 19. Effects of taiwanin C on PGE2 production induced by TPA, thapsigargin, and staurosporine. Rat peritoneal macrophages (7.5 x 10^5 cells) were incubated for 8 h in 0.5 ml of medium containing TPA (30 nM), thapsigargin (30 nM) or staurosporine (30 nM) in the presence or absence of taiwanin C (1 μM). The concentration of PGE2 in the conditioned medium was measured by radioimmunoassay. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance; $^{###}P<0.001$ vs. Non-stimulated control; $^{***}P<0.001$ vs. corresponding control.

II.3. Discussion

Taiwanin C showed no effect on the TPA-induced release of arachidonic acid from membrane phospholipids (Fig. 16) or the expression of COX-2 protein (Fig. 17), but showed a direct inhibitory effect on the activity of the isolated COX-1 and COX-2 (Fig. 18). These findings indicate that taiwanin C suppresses TPA-induced PGE_2 production by the direct inhibition of the activities of COX-1 and COX-2. Taiwanin C also inhibited COX-1-

dependent PGE_2 production (an IC_{50} value; more than 1 μM) and COX-2-dependent PGE_2 production (an IC_{50} value; 0.23 μM) (Fig. 15). It is reported that IC_{50} values change depending on the assay system [57]; probably reflecting differences in protein binding and distribution across cell membranes [58]. The mechanism of action of taiwanin C for the inhibition of PGE_2 production is similar to that of acidic NSAIDs such as indomethacin, ibuprofen, and meclofenamate.

Acidic NSAIDs commonly have a carboxylic acid residue that binds to Arg^{120} in COX-1 and blocks the approach of arachidonic acids to the catalytic subunit of COX-1 [59]. It is well known that gastrointestinal injury, a major side-effect of acidic NSAIDs [54], is caused by the inhibition of prostanoid production via the inhibition of COX-1 in the gastrointestinal mucosa. However, it has been reported that the most abundant phospholipid on the surface of the gastric mucus gel which protects the mucus from luminal acid is phosphatidylcholine, and acidic NSAIDs interact chemically with phosphatidylcholine, separate phosphatidylcholine from the surface, and induce gastrointestinal injury by exposing the surface to luminal acid [60]. Because taiwanin C has no carboxylic acid residue, it may have less gastrointestinal side-effect than acidic NSAIDs.

In order to selectively inhibit the activity of COX-2, a methylsulfonylphenyl or sulfonamoylphenyl group at the *para* position of the aryl ring must interact with the specific residues within the side-pocket of COX-2 [61]. Using this approach, several COX-2 inhibitors such as celecoxib [62] and rofecoxib [63] have been developed. However, the present study demonstrated that taiwanin C having no methylsulfonylphenyl or sulfonamoylphenyl substituent inhibits COX-2 activity. Therefore, it is suggested that a lactone moiety in the taiwanin C molecule participates in the inhibition of COX-2 activity. In the five-membered ring of some COX-2 inhibitors such as rofecoxib and celecoxib, atoms containing lone pair electrons seem to interact with some residues in COX-2. It is reported that an oxygen atom of a ketone in the lactone moiety makes a hydrogen bond with residues lining the primary COX-2 channel, particularly the Arg^{120} residue, and this interaction helps to inhibit the activity of COX-2 [64]. The inhibition of COX-2 activity by taiwanin C might involve the same mechanism.

Helioxanthin, savinin, and *cis*-dibenzylbutyrolactone that also contain a lactone moiety, inhibited the TPA-induced PGE_2 production less potently than taiwanin C (Fig. 13). The

position of the lactone moiety in these compounds is different from that in taiwanin C, and this might be the reason for their weak inhibitory effect. In contrast, *l*-sesamin having no lactone moiety showed no inhibitory effect on TPA-induced PGE_2 production (Fig. 13).

It is reported that the lignan glycoside phillyrin isolated from the leaves of *Phillyrea latifolia* (Oleaceae) inhibits PGE_2 production in calcium ionophore A23187-stimulated mouse peritoneal macrophages, and thromboxane B_2 production in A23187-stimulated human platelets with IC_{50} values of 45.6 μM and 168 μM, respectively [65], but the mechanism of action has not been clarified. In the present study, it was elucidated that the arylnaphthalide lignan taiwanin C has the direct inhibitory activity on isolated COX-1 and COX-2 with IC_{50} values of 1.06 μM and 9.31 μM, respectively (Fig. 18). In addition, it has been reported that the lignans neojusticin A and justicidin B isolated from *Justicia procumbens* L. (Acathaceae), which structurally resemble taiwanin C, inhibit arachidonic acid-induced rabbit platelet aggregation with IC_{50} values of 1.1 μM and 8.0 μM, respectively [66]. It is possible that the arachidonic acid-induced aggregation of rabbit platelets is inhibited by these lignans through the inhibition of COX activity, resulting in the inhibition of the production of thromboxane A_2 that induces platelet aggregation [67].

In conclusion, among the five lignans (*l*-sesamin, helioxanthin, savinin, taiwanin C, and *cis*-dibenzylbutyrolactone) isolated from the medicinal plant *Acanthopanax chiisanensis*, taiwanin C was the most potent inhibitor of TPA-induced PGE_2 production in rat peritoneal macrophages. Furthermore, the mechanism of its action is the direct inhibition of COX-1 and COX-2 activity. It is suggested that the anti-inflammatory activity of the extract of *Acanthopanax chiisanensis* is partly due to the inhibition of PGE_2 production by these lignans, and taiwanin C might be a lead compound for a COX inhibitor having no carboxylic acid.

Chapter III. Inhibition of production of tumor necrosis factor-α and nitrite by 2'-hydroxychalcone derivatives

III.1. Introduction

Flavonoids are the most common and widely distributed group of plant phenolic compounds, and fruits, vegetables and tea are the main dietary sources for human [68]. Flavonoids have been reported to possess a variety of biological activities, including anti-allergic, anti-inflammatory, anti-viral, and anti-carcinogenic activities [69]. It is well known that catechins in green tea, genistein in soybeans and quercetin in onions have the versatile health benefits [69].

Chalcone, one of flavonoids, is studied in terms of their various biological actions including anti-inflammatory [70], anti-tumor [71] and antioxidant effects [72]. For example, 2',5'-dihydroxychalcone and 2',3-dihydroxychalcone inhibit polymixin B-induced hind-paw edema in mice [73]. In addition, 2'-hydroxychalcone inhibits the TNF-α-induced expression of adhesion molecules such as intercellular adhesion molecules (ICAM)-1, vascular CAM (VCAM)-1 and E-selectin in human umbilical vein endothelial cells (HUVEC) [74]. Butein (3,4,2',4'-tetrahydroxychalcone) induces apoptosis by inducing caspase-3 activity and Bax expression and by reducing Bcl-2 expression in HL-60 cells [75].

Previously, we also reported that 2'-hydroxychalcone derivatives inhibit TPA-induced PGE_2 production through the suppression of COX-2 induction in rat peritoneal macrophages [76]. On the basis of these findings, to obtain further insight into the anti-inflammatory effect of 2'-hydroxychalcones, I examined the effects of various 2'-hydroxychalcone derivatives on the LPS-induced production of NO and TNF-α, and attempted to clarify the mechanism of action in cultures of RAW 264.7 cells.

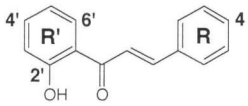
III.2. Results

III.2.1. Effects of 2'-hydroxychalcone derivatives on LPS-induced production of

nitrite and TNF-α

Production of nitrite was increased by treatment of RAW 264.7 cells with LPS (0.1 μg/ml) for 12 h, and among four derivatives (Table 3), compounds 1, 2 and 3 strongly inhibited the LPS-induced production of nitrite (Table 4). Compounds 1, 2 and 3 also suppressed the LPS-induced production of TNF-α at 6 h (Table 4). The tyrosine kinase inhibitor genistein at 30 μM inhibited the LPS-induced production of nitrite and TNF-α at 30 μM, but compound 4 showed no significant effect (Table 4).

Table 3. Chemical structures of 2'-hydroxychalcone derivatives.



Compound	R'	R
1	4'-OCH_3	H
2	4'-OCH_3	4-OH
3	6'-OCH_3	4-OH
4	4'-OCH_3	4-OCH_3

Table 4. Effects of 2'-hydroxychalcone derivatives on LPS-induced production of nitrite and TNF-α.

Treatment	Nitrite (μM)	TNF-α (pg/ml)
None	5.2 ± 0.2***	39.8 ± 2.1***
LPS (0.1 μg/mL)	32.6 ± 0.4###	208.8 ± 4.2###
LPS + Compound 1 (30 μM)	6.9 ± 0.6#***	55.0 ± 4.5#***
LPS + Compound 2 (30 μM)	7.8 ± 0.7#***	57.8 ± 2.9##**
LPS + Compound 3 (30 μM)	9.2 ± 0.4####	57.4 ± 2.9##**
LPS + Compound 4 (30 μM)	31.5 ± 0.4###	202.7 ± 5.1###
LPS + Genistein (30 μM)	15.9 ± 0.6###	55.7 ± 2.8##**

Values are the means from four samples with the S.E.M. Statistical significance; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. None; $^{***}P<0.001$ vs. LPS control.

III.2.2. Effects of 2'-hydroxy-4'-methoxychalcone (compound 1) on LPS-induced expression of iNOS and production of nitrite

Incubation with LPS (0.1 μg/ml) for 12 h markedly induced iNOS expression and increased nitrite production (Fig. 21A and B). Under these conditions, compounds 1 inhibited the LPS-induced expression of iNOS and production of nitrite in a concentration-dependent manner at 3 to 30 μM (Fig. 21A and B). These findings indicate that compounds 1 inhibits the LPS-induced production of nitrite through the inhibition of iNOS expression.

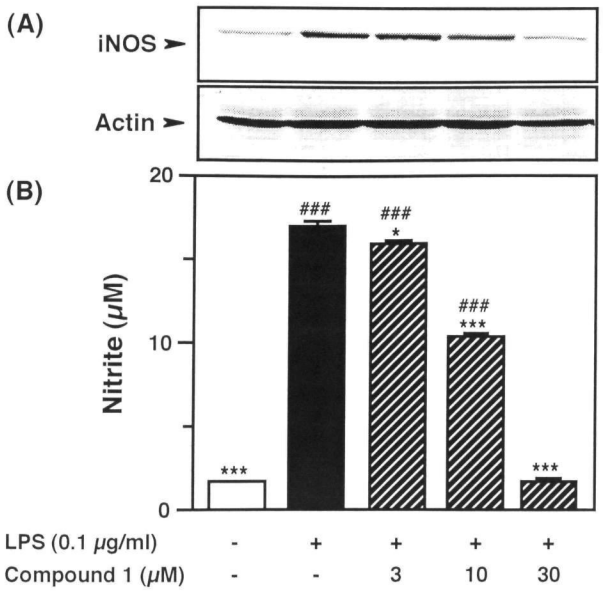


Figure 21. Effects of 2'-hydroxy-4'-methoxychalcone (compound 1) on LPS-induced expression of iNOS and production of nitrite. RAW 264.7 cells (5 x 10^5 cells/ml) were incubated for 12 h in 2 ml (A) or 0.5 ml (B) of medium containing LPS (0.1 μg/ml) and the indicated concentration of compound 1. (A) The protein levels of iNOS and actin were determined by immunoblotting. (B) The concentration of nitrite in the conditioned medium was measured using Griess reagent. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance; $^{\#\#\#}P<0.001$ vs. Non-stimulated control; $^{*}P<0.05$, $^{***}P<0.001$ vs. LPS control.

III.2.3. Effects of 2'-hydroxy-4'-methoxychalcone (compound 1) on LPS-induced increase in the level of TNF-α mRNA and protein

Treatment with LPS (0.1 μg/ml) increased the level of TNF-α mRNA at 4 h (Fig. 22A) and TNF-α protein at 6 h (Fig. 22B), which were lowered by compound 1 at 3 to 30 μM in a concentration-dependent manner. These findings indicate that the inhibition of TNF-α production by compounds 1 is due to the suppression of the LPS-induced expression of TNF-α mRNA.

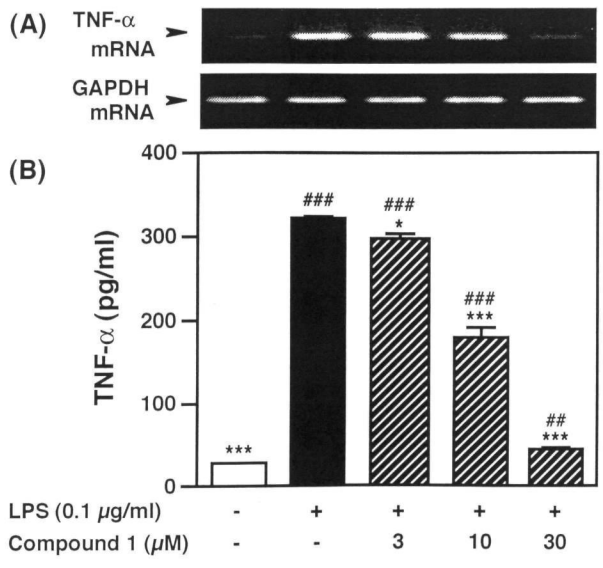


Figure 22. Effects of 2'-hydroxy-4'-methoxychalcone (compound 1) on LPS-induced expression of TNF-α. RAW 264.7 cells (5 x 10^5 cells/ml) were incubated for 4 h (A) or 6 h (B) in 1 ml (A) or 0.5 ml (B) of medium containing LPS (0.1 μg/ml) and the indicated concentration of compound 1. (A) The levels of mRNA for TNF-α and GAPDH were determined by RT-PCR. (B) The concentration of TNF-α in the conditioned medium was measured by ELISA. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. Non-stimulated control; $^{*}P<0.05$, $^{***}P<0.001$ vs. LPS control.

III.2.4. Effects of 2'-hydroxy-4'-methoxychalcone (compound 1) on LPS-induced activation of NF-κB and AP-1

Treatment with LPS (0.1 μg/ml) for 1 h increased the activation of both NF-κB (Fig. 23A) and AP-1 (Fig. 23B). In the presence of compound 1 at 3 to 30 μM, the activation of NF-κB and AP-1 was suppressed (Fig. 23A and 23B). These findings indicate that the inhibition of the LPS-induced production of nitrite and TNF-α by compound 1 is induced by the suppression of activation of NF-κB and AP-1.

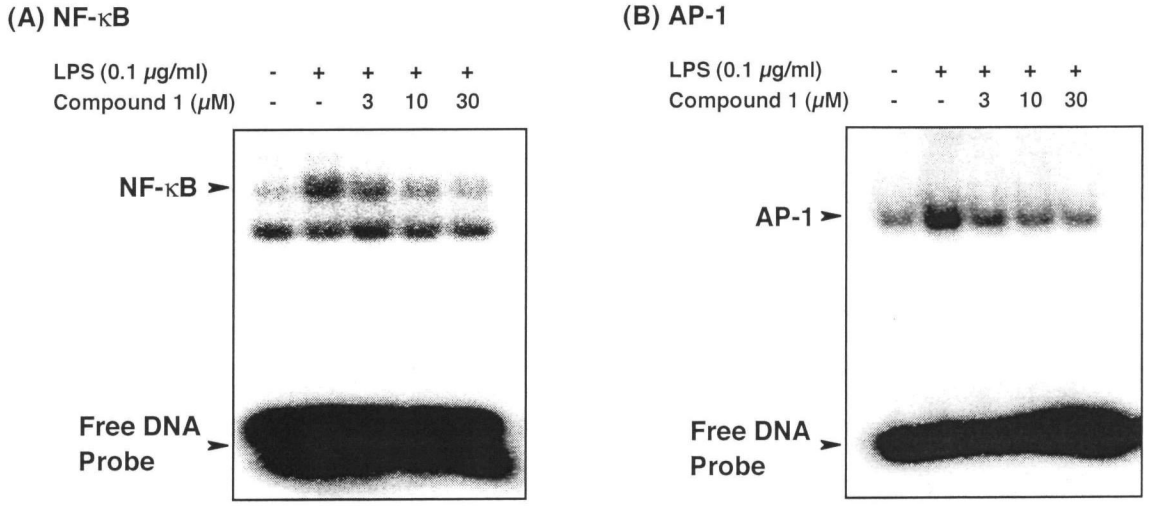


Figure 23. Effects of 2'-hydroxy-4'-methoxychalcone (compound 1) on LPS-induced activation of NF-κB and AP-1. RAW 264.7 cells (5 x 10^5 cells/ml) were incubated for 1 h in 4 ml of medium containing LPS (0.1 μg/ml) and the indicated concentration of compound 1. The nuclear protein was extracted, and the levels of NF-κB (A) and AP-1 (B) bound to the DNA probe was determined by EMSA.

III.2.5. Effects of 2'-hydroxy-4'-methoxychalcone (compound 1) on LPS-induced degradation of IκB-α

Incubation of RAW 264.7 cells for 20 min in the presence of LPS (0.1 μg/ml) induced degradation of IκB-α, which was significantly inhibited by compound 1 at 3 to 30 μM (Fig. 24). These findings indicate that compound 1 suppresses the activation of NF-κB through the inhibition of the LPS-induced degradation of IκB-α.

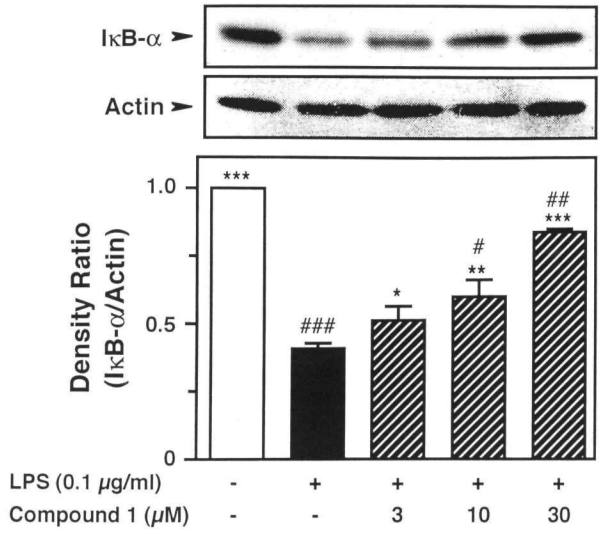


Figure 24. Effects of 2'-hydroxy-4'-methoxychalcone (compound 1) on LPS-induced degradation of IκB-α. RAW 264.7 cells (5 x 10^5 cells/ml) were incubated for 20 min in 2 ml of medium containing LPS (0.1 μg/ml) and the indicated concentration of compound 1. The protein levels of IκB-α and actin were determined by immunoblotting. The density ratios of IκB-α to actin were calculated, and the value of the density ratio in the non-treated control group is set to 1.0. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. Non-stimulated control; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. LPS control.

III.2.6. Effects of 2'-hydroxy-4'-methoxychalcone (compound 1) on LPS-induced phosphorylation of JNK and c-Jun

After incubation with LPS (0.1 μg/ml) for 30 min, phosphorylation of JNK and c-Jun was significantly increased (Fig. 25A and B). Compound 1 inhibited the LPS-induced phosphorylation of JNK (Fig. 25A) and c-Jun (Fig. 25B) in a concentration-dependent manner, at which concentrations the LPS-induced activation of AP-1 was inhibited (Fig. 23B). These findings indicate that compound 1 down-regulates the c-Jun phosphorylation via the suppression of JNK phosphorylation.

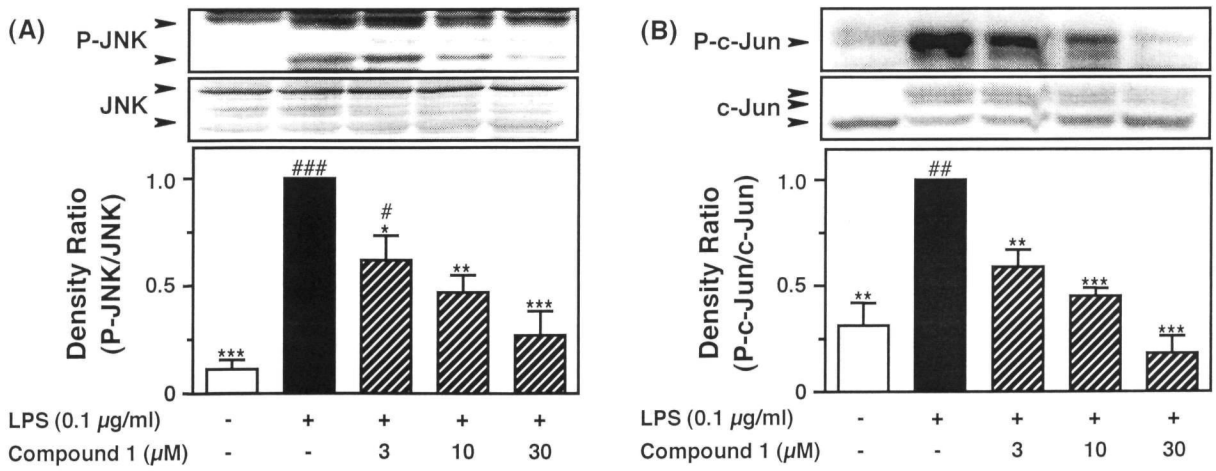


Figure 25. Effects of 2'-hydroxy-4'-methoxychalcone (compound 1) on LPS-induced phosphorylation of JNK and c-Jun. RAW 264.7 cells (5 x 10^5 cells/ml) were incubated for 30 min in 2 ml of medium containing LPS (0.1 μg/ml) and the indicated concentration of compound 1. The protein levels of phosphorylated (P)-JNK, JNK, phosphorylated (P)-c-Jun and c-Jun were determined by immunoblotting. The density ratios of P-JNK to JNK and P-c-Jun to c-Jun were calculated, and the value of the density ratio in the LPS control group is set to 1.0. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. Non-stimulated control; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. LPS control.

III.3. Discussion

In cultures of RAW 264.7, three 2'-hydroxychalcone derivatives, compounds 1, 2 and 3, suppressed the LPS-induced production of nitrite and TNF-α, but compound 4 showed no effect (Table 2). These findings indicated that the substitution of -OH with $-OCH_3$ at position 4 (compound 4) decreased the inhibitory activity of compound 2. The reduction of the inhibitory activity caused by the substitution of 4-OH with $4\text{-}OCH_3$ was also observed in our previous study on the suppression of TPA-induced PGE_2 production in rat peritoneal macrophages [76]. These findings suggested that the inhibition of LPS-induced production of nitrite and TNF-α by 2'-hydroxychalcone derivatives is induced by a similar mechanism to the inhibition of TPA-induced PGE_2 production.

Similar to the effect of imperatorin described in Chapter I, compound 1 inhibited the LPS-induced activation of NF-κB (Fig. 23A). Therefore, it is suggested that the suppressive effects of compound 1 on the production of nitrite, TNF-α and PGE_2 were meditated by the inhibition of NF-κB activation. Compound 1 suppressed the activation of NF-κB via the inhibition of the LPS-induced degradation of IκB-α (Fig. 24), while imperatorin suppressed that by direct inhibition of NF-κB binding to DNA (Fig. 10 in Chapter I). In non-activated cells, NF-κB is binding with the inhibitory protein IκB-α, thereby prevented its nuclear translocation [41]. Upon LPS stimulation, IκB kinase (IKK)-β is activated and induces phosphorylation of IκB-α, resulting in its ubiquitination and degradation by proteasome pathway [41]. Therefore, it is suggested that the inhibition by compound 1 of NF-κB activation is mediated through suppression of the upstream signaling pathway of IκB-α degradation. Besides NF-κB, it is reported that the promoter region of both *iNOS* and *TNF-α* genes also contains a binding site for the transcription factor AP-1 [77,78]. Furthermore, activation of JNK leads to the phosphorylation of serine 63 and 73 in c-Jun, a component of AP-1, and increases the transcriptional activity of AP-1 [79], indicating that the activation of AP-1 is mainly dependent on the activation of the JNK signaling pathway. Therefore, the inhibition by compound 1 of AP-1 activation (Fig. 23B) through reduction of JNK phosphorylation (Fig. 25A) indicates that compound 1 inhibits upstream kinase of JNK.

It is reported that the redox regulation is involved in the activation of NF-κB and AP-1 [80,81], and the antioxidant reagent pyrrolidine dithiocarbamate and *N*-acetylcysteine inhibit the activation of NF-κB and AP-1 [82,83]. Moreover, these inhibitors strongly suppress the production of nitrite and TNF-α [82,84]. It is also reported that 2'-hydroxychalcones have a potent antioxidant activity [85]. Therefore, it is possible that the 2'-hydroxychalcone derivative inhibited the activation of NF-κB and AP-1 through its antioxidant property. There is another possible mechanism of 2'-hydroxychalcone derivative for the inhibition of NF-κB and AP-1. It is reported that various flavonoids including 2'-hydroxychalcone [86], genistein [87] and quercetin [88] have inhibitory activities against protein tyrosine kinases. Because activations of tyrosine kinase such as c-Src by LPS lead to both activation of NF-κB and phosphorylation of MAPKs, it is possible that the inhibition of NF-κB and AP-1 by 2'-hydroxychalcone derivative is mediated through its inhibitory activities against protein tyrosine kinases. Furthermore, it is reported that 2'-hydroxychalcones such as butein (3,4,2',4'-tetrahydroxychalcone) [89] and ON-III (2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone) [90] inhibited tyrosine kinase activity of epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR), respectively. Therefore, the anti-tumor activity of 2'-hydroxychalcone also might be induced by inhibition of tyrosine kinases.

In conclusion, 2'-hydroxychalcone derivatives suppress the LPS-induced production of nitrite and TNF-α by inhibiting the activation of both NF-κB and AP-1. Because NF-κB and AP-1 are critical transcription factors that regulate the production of various proinflammatory proteins and cytokines in activated macrophages during the process of inflammation, the inhibition of these transcription factors might be an effective therapeutic approach for inflammatory diseases. Taken together, it is possible that 2'-hydroxy-4'-methoxychalcone (compound 1) is a lead compound for novel anti-inflammatory drugs having inhibitory activity on the production of various inflammatory mediators such as PGE_2, NO and cytokines.

Chapter IV. Inhibition by resveratrol of FcεRI-mediated activation of RBL-2H3 cells

IV.1. Introduction

Resveratrol (*trans*-3,4',5-trihydroxystilbene) (Fig. 26) is a natural polyphenolic compound presents in various plants, including red grapes, berries and peanuts [91]. Resveratrol is identified as a phytoalexin and synthesized in response to injury, fungal infection or ultraviolet light [92]. Until now, various biological activities of resveratrol have been reported, and dietary consumption of resveratrol is thought to provide beneficial effects on various diseases. Resveratrol prevented the oxidation of low-density lipoprotein through its anti-oxidative activity and reduced the risk of coronary artery disease [93,94]. Moreover, resveratrol was shown to have cancer chemopreventive activity [95], anti-platelet aggregation activity [96], and neuroprotective activity [97].

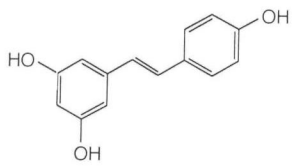


Figure 26. Chemical structure of resveratrol.

One of the beneficial biological activities of resveratrol is anti-inflammatory activity. Resveratrol inhibited LPS-induced expression of iNOS and COX-2 in macrophages [98], and TNF-α-induced expression of VCAM-1 and ICAM-1 in HUVEC [99]. Resveratrol also showed suppressive activities against expression of proinflammatory cytokines such as IL-8, IL-1β and TNF-α via its inhibitory effects on NF-κB and AP-1 [100]. Moreover, it has been reported that resveratrol and other stilbene compounds including rhapontigenin and piceatannol isolated from the rhizome of *Rheum undulatum* inhibited the release of histamine in antigen-stimulated rat peritoneal mast cells [101]. However, the action mechanism of resveratrol has not been elucidated. To clarify the mechanism of resveratrol for the suppression of the activation of mast cells, I have analyzed effects of resveratrol on the antigen-induced degranulation, production of IL-13 and LTC_4, and phosphorylation of several protein kinases in IgE-sensitized rat basophilic leukemia RBL-2H3 cells.

IV.2. Results

IV.2.1. Effects of resveratrol on antigen-induced degranulation of RBL-2H3 cells

Stimulation with the antigen dinitrophenol-conjugated human serum albumin (DNP-HAS, 50 ng/ml) significantly increased the release of hexosaminidase at 15 min (Fig. 27). Resveratrol at 10 to 100 μM suppressed the antigen-induced release of hexosaminidase in a concentration-dependent manner, and the PI3K inhibitor wortmannin at 100 nM strongly inhibited the release to the same extent as resveratrol at 100 μM (Fig. 27). These findings indicate that resveratrol inhibits the antigen-induced degranulation of RBL-2H3 cells.

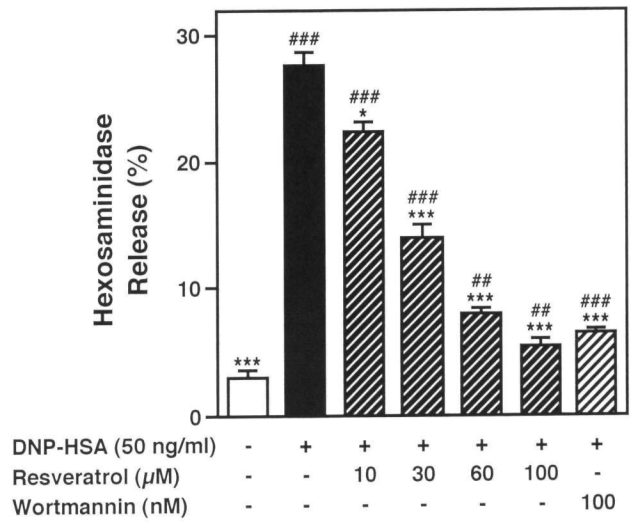


Figure 27. Effects of resveratrol on antigen-induced degranulation of RBL-2H3 cells. IgE-sensitized RBL-2H3 cells (5 x 10^5 cells/ml) were stimulated for 15 min in 0.5 ml of medium containing DNP-HSA (50 ng/ml) and the indicated concentration of resveratrol or wortmannin. The amount of hexosaminidase released was determined, and the percentage released was calculated. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. Non-stimulated control; $^{*}P<0.05$, $^{***}P<0.001$ vs. DNP-HSA control.

IV.2.2. Effects of resveratrol on antigen-induced phosphorylation of Akt

Treatment of the cells with the antigen for 15 min induced phosphorylation of Akt, and resveratrol suppressed the antigen-induced phosphorylation of Akt (Fig. 28) at which concentrations the antigen-induced degranulation was inhibited (Fig. 27). Wortmannin at 100 nM also suppressed the antigen-induced phosphorylation of Akt to a lower level than that of the un-stimulated cells (Fig. 28). These findings suggest that the inhibition of the antigen-induced degranulation of mast cells by resveratrol is mediated through suppression of the PI3K pathway.

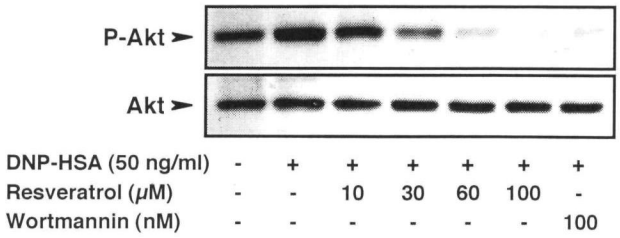


Figure 28. Effects of resveratrol on antigen-induced phosphorylation of Akt. IgE-sensitized RBL-2H3 cells (5 x 10^5 cells/ml) were stimulated for 15 min in 2 ml of medium containing DNP-HSA (50 ng/ml) and the indicated concentration of resveratrol or wortmannin. The protein levels of phosphorylated (P)-Akt and Akt were determined by immunoblotting.

IV.2.3. Effects of resveratrol on antigen-induced production of IL-13

Levels of IL-13 mRNA at 2 h and protein at 4 h were increased by the antigen treatment, and resveratrol lowered the antigen-induced increase in the levels of IL-13 mRNA (Fig. 29A) and protein (fig. 29B) in a concentration-dependent manner. The JNK inhibitor SP600125 (30 μM) also suppressed the antigen-induced increase in the levels of IL-13 mRNA and protein (Fig. 29A and B). These findings indicate that resveratrol inhibits IL-13 production by decreasing the levels of IL-13 mRNA.

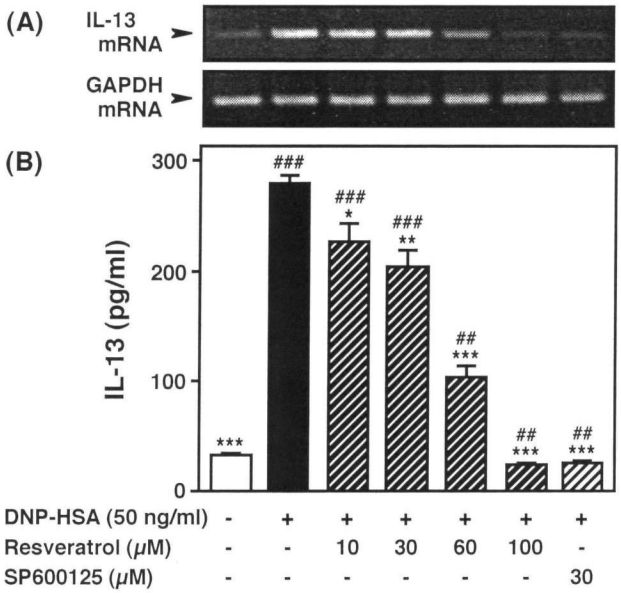


Figure 29. Effects of resveratrol on antigen-induced expression of IL-13. IgE-sensitized RBL-2H3 cells (5 x 10^5 cells/ml) were stimulated for 2 h (A) or 4 h (B) in 1 ml (A) or 0.5 ml (B) of medium containing DNP-HSA (50 ng/ml) and the indicated concentration of resveratrol or SP600125. (A) The levels of mRNA for IL-13 and GAPDH were then determined by RT-PCR. (B) The concentration of IL-13 in the conditioned medium was measured by ELISA. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. Non-stimulated control; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. DNP-HSA control.

IV.2.4. Effects of resveratrol on antigen-induced activation of AP-1

The antigen treatment for 1 h increased the binding of AP-1 to its specific oligonucleotide probe, and resveratrol at 10 to 100 μM inhibited the binding of AP-1 (Fig. 30A).

Resveratrol also suppressed the antigen-induced phosphorylation of c-Jun, a component of AP-1 (Fig. 30B). Almost complete inhibition was observed on treatment with resveratrol at

100 μM or the JNK inhibitor SP600125 at 30 μM (Fig. 30A and B). These findings indicate that resveratrol inhibits the antigen-induced production of IL-13 by suppressing the activation of AP-1.

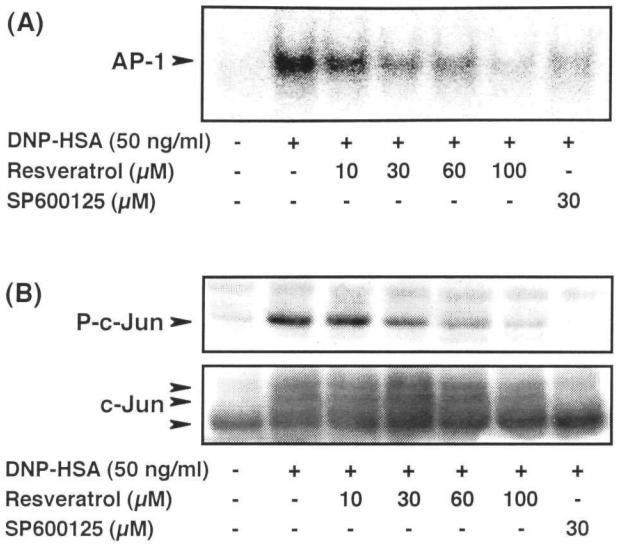


Figure 30. Effects of resveratrol on antigen-induced activation of AP-1 and phosphorylation of c-Jun. IgE-sensitized RBL-2H3 cells (5 x 10^5 cells/ml) were stimulated for 1 h (A) or 45 min (B) in 4 ml (A) or 2 ml (B) of medium containing DNP-HSA (50 ng/ml) and the indicated concentration of resveratrol or SP600125. (A) The nuclear protein was extracted, and the levels of AP-1 bound to the DNA probe was determined by EMSA. (B) The protein levels of phosphorylated (P)-c-Jun and c-Jun were determined by immunoblotting.

IV.2.5. Effects of resveratrol on antigen-induced production of LTC_4

Treatment with the antigen for 30 min significantly induced the production of LTC_4, and resveratrol at 10 to 100 μM suppressed the production in a concentration-dependent manner (Fig. 31). The MEK inhibitor PD98059 at 50 μM also suppressed the antigen-induced LTC_4 production to almost the same level as that on treatment with resveratrol at 60 μM (Fig. 31).

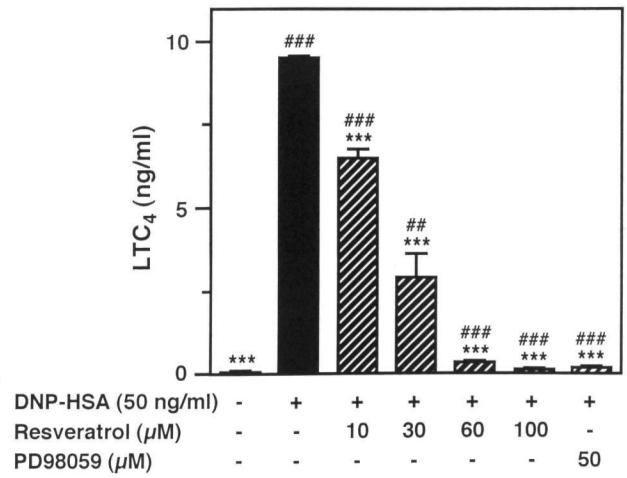


Figure 31. Effects of resveratrol on antigen-induced production of LTC4. IgE-sensitized RBL-2H3 cells (5 x 10^5 cells/ml) were stimulated for 30 min in 0.5 ml of medium containing DNP-HSA (50 ng/ml) and the indicated concentration of resveratrol or PD98059. The concentration of LTC4 in the conditioned medium was determined by EIA. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. Non-stimulated control; $^{***}P<0.001$ vs. DNP-HSA control.

IV.2.6. Effects of resveratrol on antigen-induced release of arachidonic acid and phosphorylation of ERK

Treatment with the antigen for 30 min increased the release of radioactivity from [^{3}H]arachidonic acid-labeled RBL-2H3 cells, and in the presence of resveratrol, a concentration-dependent inhibition was observed at 10 to 100 μM (Fig. 32A). Phosphorylation of ERK was induced 5 min after the antigen treatment, and was inhibited by resveratrol at 10 to 100 μM in a concentration-dependent manner (Fig. 32B). PD98059 at 50 μM suppressed the antigen-induced release of arachidonic acid parallel to its inhibition of the phosphorylation of ERK (Fig. 32A and B). These findings suggest that the inhibition by resveratrol of the antigen-induced release of arachidonic acid and production of LTC_4 is due to the inhibition of phosphorylation of ERK which activates PLA_2.

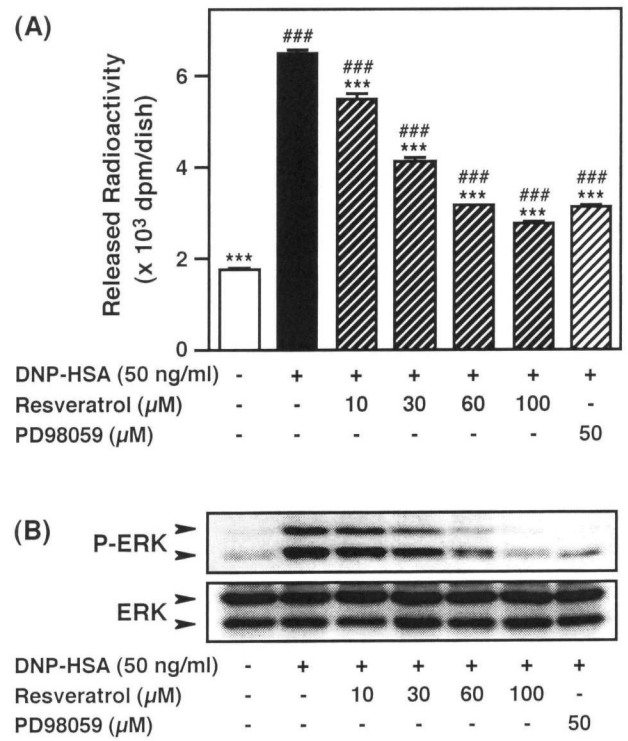


Figure 32. Effects of resveratrol on antigen-induced release of arachidonic acid and phosphorylation of ERK. IgE-sensitized RBL-2H3 cells (5 x 10^5 cells/ml) were stimulated for 30 min (A) or 5 min (B) in 0.5 ml (A) or 2 ml (B) of medium containing DNP-HSA (50 ng/ml) and the indicated concentration of resveratrol or PD98059. (A) The amount of radioactivity released into the conditioned medium from [^{3}H]arachidonic acid-labeled RBL-2H3 cells was determined. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance; $^{###}P<0.001$ vs. Non-stimulated control; $^{***}P<0.001$ vs. DNP-HSA control. (B) The protein levels of phosphorylated (P)-ERK and ERK were determined by immunoblotting.

IV.2.7. Effects of resveratrol on antigen-induced tyrosine phosphorylation of Syk and Pyk2

As shown in Fig. 33A and B, the antigen treatment for 5 min induced tyrosine phosphorylation of Syk parallel to the phosphorylation of Pyk2, a downstream kinase directly phosphorylated by Syk [102]. At 10 to 100 μM, both resveratrol and the Syk inhibitor

piceatannol suppressed the antigen-induced tyrosine phosphorylation of Syk and Pyk2 (Fig. 33A and B).

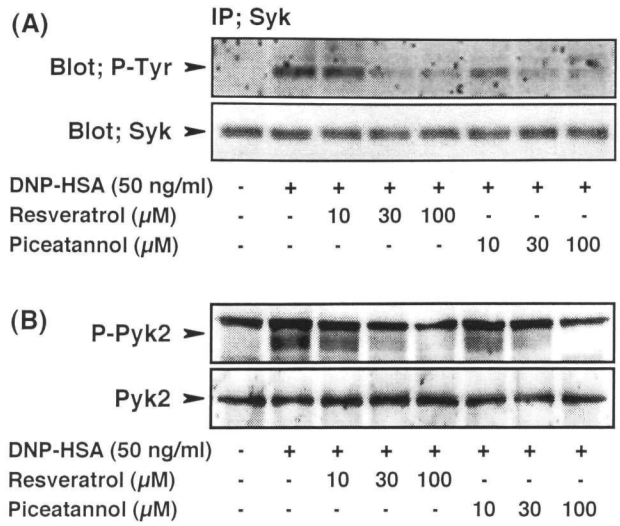


Figure 33. Effects of resveratrol on antigen-induced tyrosine phosphorylation of Syk and Pyk2. IgE-sensitized RBL-2H3 cells (5 x 10^5 cells/ml) were stimulated for 5 min in 10 ml of medium containing DNP-HAS (50 ng/ml) and the indicated concentration of resveratrol and piceatannol. (A) Syk was immunoprecipitated (IP) and the protein levels of tyrosine-phosphorylated (P-Tyr) Syk and Syk were determined by immunoblotting. (B) The protein levels of phosphorylated (P)-Pyk2 and Pyk2 were determined by immunoblotting.

IV.2.8. Effects of piceatannol on antigen-induced phosphorylation of Akt, c-Jun and ERK

To confirm whether piceatannol also inhibits the activation of downstream kinases, effects of piceatannol on the antigen-induced phosphorylation of Akt, c-Jun and ERK were examined. As shown in Fig. 34, piceatannol also inhibited the antigen-induced phosphorylation of Akt, c-Jun and ERK with a similar potency to that of resveratrol. These findings indicate that resveratrol inhibits the antigen-induced phosphorylation of Akt, c-Jun and ERK by suppressing the activation of their upstream kinase Syk.

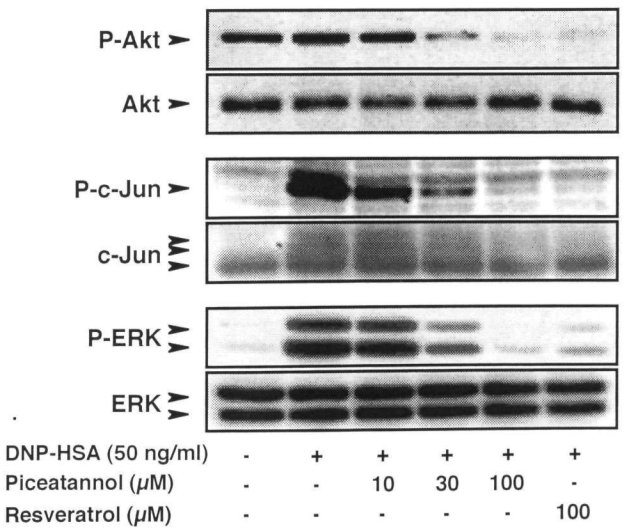


Figure 34. Effects of piceatannol on antigen-induced phosphorylation of Akt, c-Jun and ERK. IgE-sensitized RBL-2H3 cells (5 x 10^5 cells/ml) were stimulated for 5 min (ERK), 15 min (Akt) or 45 min (c-Jun) in 2 ml of medium containing DNP-HAS (50 ng/ml) and the indicated concentration of piceatannol or resveratrol. The protein levels of phosphorylated (P)-Akt, Akt, phosphorylated (P)-c-Jun, c-Jun, phosphorylated (P)-ERK and ERK were determined by immunoblotting.

IV.3. Discussion

The antigen stimulation induced the release of three kinds of inflammatory mediators from RBL-2H3 cells; granule-stored mediator hexosaminidase (Fig. 27), cytokine IL-13 (Fig. 29) and arachidonate metabolite LTC_4 (Fig. 31), which were inhibited by resveratrol in a concentration-dependent manner.

It is reported that the promoter region of the *IL-13* gene contains binding sites for AP-1, GATA-3 and NF-AT [103]. Moreover, it is reported that the JNK inhibitor SP600125 completely suppressed the antigen-induced production of IL-13 in RBL-2H3 cells [104], suggesting that AP-1 acts as a critical transcription factor for IL-13 expression. Phosphorylation of JNK leads to an increase in the transcriptional activity of AP-1 through the phosphorylation of c-Jun [79]. The antigen-induced phosphorylation of JNK was also inhibited by resveratrol at which concentrations the antigen-induced phosphorylation of c-Jun was inhibited (data not shown). These findings indicate that resveratrol inhibits the antigen-induced expression of IL-13 by blocking the JNK pathway. Because PI3K is involved in not only FcεRI-mediated degranulation [105], but also the activation of the JNK pathway in mast cells [106], the inhibition by resveratrol of the antigen-induced activation of the PI3K pathway might account for the suppression of degranulation and activation of AP-1.

It is reported that the stimulation of IgE-sensitized RBL-2H3 cells with antigen rapidly induces production of eicosanoids including LTB_4, LTC_4, LTE_4 and PGD_2, the major product being LTC_4 [107]. Resveratrol inhibited the antigen-induced production of LTC_4 and release of arachidonic acid in mast cells (Figs. 31 and 32A) in parallel with suppression of the phosphorylation of ERK (Fig. 32B) that activates PLA_2. To clarify whether resveratrol also suppresses activation of ERK through the inhibition of PI3K, the effect of wortmannin on the activation of ERK was examined, but the antigen-induced phosphorylation of ERK was not affected by wortmannin at 10 to 100 nM (data not shown). This finding indicates that the antigen-induced activation of ERK is not involved in the PI3K pathway as previously reported [108]. Therefore, it is suggested that resveratrol might inhibit the upstream kinase of both PI3K and ERK.

The activation of protein tyrosine kinases such as the Src family kinases Lyn and Fyn, and

the Syk/Zap family kinase Syk is one of the earliest signaling events induced by the aggregation of FcεRI on mast cells [109]. Among these protein tyrosine kinases, subsequent trans- and auto-phosphorylations of Syk result in an increase in catalytic activity and consequential tyrosine phosphorylation of downstream signaling kinases [109]. Based on structural similarity to the Syk inhibitor piceatannol (*trans*-3,3',4,5'-tetrahydroxystilbeben) which has one more hydroxy group on 3'-position than resveratrol (*trans*-3,4,5'-tetrahydroxystilbeben), I postulated that one possible mechanism by which resveratrol inhibits mast cell activation is the inhibition of Syk. As shown in Fig. 33, resveratrol and piceatannol inhibited the antigen-induced phosphorylation of Syk and its downstream kinase Pyk2. Furthermore, piceatannol also suppressed activation of downstream kinases that were inhibited by resveratrol (Fig. 34). These findings suggest that the action of resveratrol is almost the same as that of piceatannol in RBL-2H3 cells.

In Syk-deficient mast cells, antigen treatment did not induce tyrosine phosphorylation of cellular proteins, degranulation, or the mobilization of calcium [110]. It is reported that Syk inhibitors including piceatannol, ER-27319, and BAY61-3606 down-regulated the FcεRI-mediated signaling in mast cells [111]. Moreover, oral administration of BAY61-3606 to rats suppressed antigen-induced bronchoconstriction, bronchial edema and airway inflammation [112]. Therefore, Syk is suggested to play a critical role in the antigen-induced activation of mast cells, and inhibition of Syk may be a novel target for the treatment of allergic inflammation.

In conclusion, resveratrol inhibits the antigen-induced activation of mast cells including degranulation, IL-13 expression and LTC_4 production by inhibiting the activation of protein kinases Akt, JNK and ERK, respectively. Furthermore, the inhibition by resveratrol of these protein kinases is mediated by suppression of the phosphorylation of Syk. Taken together, it is suggested that resveratrol has therapeutic benefits in prevention and treatment for both early phase (degranulation and eicosanoids production) and late phase (cytokines production) in mast cell-mediated inflammatory responses.

Summary

In this study, I have analyzed the inhibitory effects of four kinds of natural products, furanocoumarins isolated from *Angelica dahurica*, lignans isolated from *Acanthopanax chiisanensis*, synthesized flavonoids 2'-hydroxychalcone derivatives, and the stilbene resveratrol on the production of inflammatory chemical mediators and cytokines in activated macrophages and mast cells, and clarified their mechanisms of action.

Among the isolated furanocoumarins and their semi-synthesized compounds, I have found that phellopterin, imperatorin and isoimperatorin potently inhibited the LPS-induced PGE_2 production in macrophages, and the inhibition by imperatorin is mediated by suppression of the induction of COX-2 and mPGES-1. Imperatorin also inhibited the LPS-induced TNF-α production via suppression of NF-κB activation, without affecting the activations of MAPKs. The suppression by imperatorin of NF-κB activation is due to the direct inhibition of binding of NF-κB to DNA. These findings indicate that the inhibitory effects of imperatorin on activation of macrophages are induced by suppression of NF-κB activation (Chapter I).

Among the isolated five lignans, I have found that taiwanin C showed the most potent inhibitory effect on TPA-induced PGE_2 production in macrophages. TPA-induced release of radioactivity from [^{3}H]arachidonic acid-labeled macrophages and expression of COX-2 were not affected by taiwanin C, but the activities of isolated COX-1 and -2 were inhibited. These findings suggested that taiwanin C inhibits PGE_2 production by the direct inhibition of COX activity similar to NSAIDs (Chapter II).

I have found that the synthesized four 2'-hydroxychalcone derivatives showed suppressive effects on the LPS-induced production of TNF-α and nitrite. Among them, 2'-hydroxy-4'-methoxychalcone (compound 1) inhibited the LPS-induced activation of NF-κB and AP-1 through the suppression of the LPS-induced IκB-α degradation and JNK phosphorylation, respectively. These findings indicated that the inhibition by 2'-hydroxychalcone derivatives

of TNF-α and nitrite production is mediated by the inhibition of NF-κB and AP-1 activation (Chapter III).

I have found that resveratrol has suppressive effects on the antigen-induced degranulation and production of IL-13 and LTC_4 in RBL-2H3 cells. Resveratrol inhibited the antigen-induced phosphorylation of Syk and its downstream kinases including Akt, c-Jun and ERK. These findings indicated that resveratrol inhibits the activation of mast cells by suppressing the antigen-induced tyrosine phosphorylation of Syk (Chapter IV).

Taken together, it is suggested that imperatorin, taiwanin C and 2'-hydroxy-4'-methoxychalcone (compound 1), and resveratrol might be lead compounds for novel anti-inflammatory drugs having suppressive effects on the activation of macrophages, and mast cells, respectively. In addition to the further clarification of their mechanism of action for anti-inflammatory activity of these compounds, it is expected that a better understanding of the structure-activity relationship, the drug metabolism and the molecular modeling may provide the approaches to the development of the new anti-inflammatory drugs.

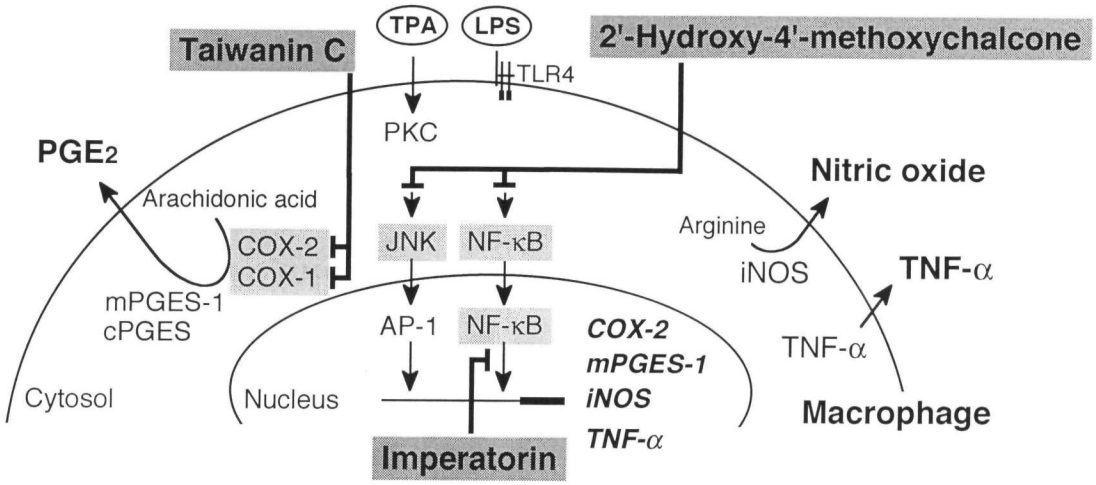


Figure 35. Inhibition by imperatorin, taiwanin C and 2'-hydroxy-4'-methoxychalcone of macrophage activation.

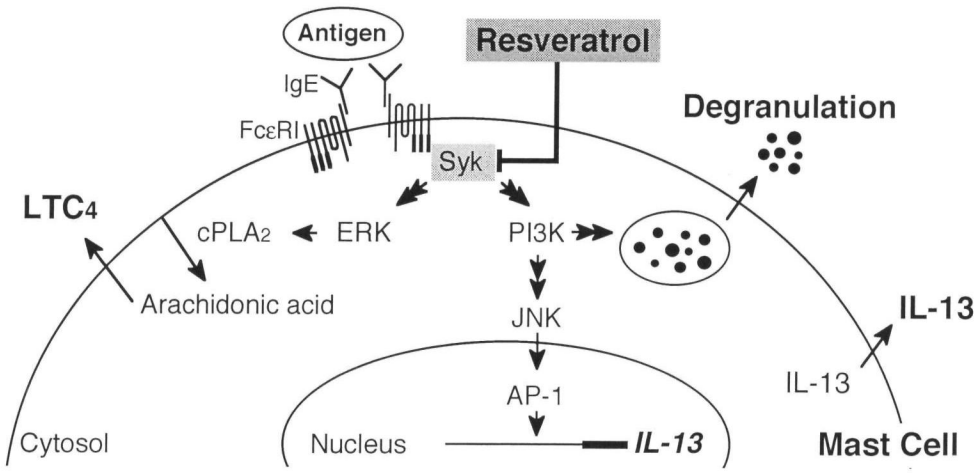


Figure 36. Inhibition by resveratrol of mast cell activation.

Materials and methods

Isolation of furanocoumarins from the roots of *Angelica dahurica*

The roots of *Angelica dahurica* were purchased from the local market (Kyung-Dong-Si-Jang) in Seoul, Korea in September 2000 and were botanically identified by Prof. H. J. Chi at Natural Products Research Institute, Seoul National University, Korea where the voucher specimen has been deposited (Voucher No: NPRI-2013). The coarsely powdered plant material (3 kg) was extracted three times with methanol under reflux over a total 4 h period. The resultant extracts were combined and concentrated under reduced pressure to afford 520 g of residue. The methanol extract was partitioned between *n*-hexane:methanol:water (10:1:9), and the residue was then partitioned between ethyl acetate and water. Column chromatography of the *n*-hexane fraction (30.4 g) over silica gel (70-230 mesh, Art, 7734 Merch) using *n*-hexane-diethylether as a stepwise gradient elution (0-100% of diethylether) gave phellopterin (0.98 g), imperatorin (2.88 g) and isoimperatorin (1.68 g). Column chromatography of the ethyl acetate fraction (75.3 g) over silica gel (70-230 mesh, Art, 7734 Merch) using the methylene chloride-methanol as a stepwise gradient elution (0-20% of methanol) gave byakangelicin (3.69 g), $[\alpha]_D$: +17.5° (*c* 0.10, C_5H_5N) and oxypeucedanin methanolate (0.11 g), $[\alpha]_D$: +18.0° (*c* 0.20, $CHCl_3$). The five compounds isolated from the dried roots of *Angelica dahurica* (Fig. 1) were identified by comparison of the spectral data (NMR, IR, UV) with authentic compounds.

Semi-synthesization of derivatives from the isolated furanocoumarins

By the semi-synthetic methods, 2',3'-dihydrobyakangelicin, $[\alpha]_D$: +18.5° (*c* 0.30, C_5H_5N), 2',3',3,4-tetrahydrobyakangelicin, $[\alpha]_D$: +17.5° (*c* 0.30, C_5H_5N), 2',3'-dihydro-8-hydroxybergapten, 2',3'-dihydro-8-hydroxypsoralen and 2',3'-dihydrooxypeucedanin methanolate $[\alpha]_D$: +19.0° (*c* 0.25, $CHCl_3$) were synthesized (Fig. 2). Furanocoumarins have one double bond in each furan ring and pyrano-2-one ring, and a carbon-carbon double bond of furanocoumarins is selectively hydrogenated by the regulation of a molar ratio of 10% palladium/charcoal (Pd/C). For the selective hydrogenation of a double bond in a furan ring, Pd/C and furanocoumarin at 1:1 molar ratio were dissolved in ethanol (1 g/50 ml) and

vigorously mixed for 30 min at room temperature under H_2 gas (1 atm). For the hydrogenation of the two double bonds, Pd/C and furanocoumarin were dissolved in ethanol at 2:1 molar ratio, and hydrogenated as described above. In case of phellopterin and imperatorin, the side chain of furanocoumarin, 2,3-dihydroxy-3-methyl-butoxyl, was removed by the Pd/C catalytic hydrogenation process.

Isolation of lignans from the roots of *Acanthopanax chiisanensis*

The roots of *Acanthopanax chiisanensis* were collected at Kong Ju, Korea and the voucher specimen (Voucher No: Shin 9910-2) was deposited in the Herbarium of Natural Products Research Institute, Seoul National University, Korea. The air-dried powdered roots (1.5 kg) were extracted three times with methanol (3 x 3,000 ml) under reflux. The extracts (159 g) thus obtained were partitioned between equal volume of *n*-hexane (42 g) and water, and the aqueous layer was again partitioned successively with chloroform (26 g), ethyl acetate (10 g) and *n*-butanol (24 g). Repeated silica gel column chromatography (7 x 60 cm) of the chloroform fraction eluting with *n*-hexane and ethyl acetate solvent system (gradient elution) gave *l*-sesamine (3,4:3',4'-bis(methylenedioxy)-7,9':7',9-diepoxylignan, 26 mg), $[\alpha]_D$: -87.2° (*c* 1.08, $CHCl_3$), helioxanthin (3,4:3',4'-bis(methylenedioxy)-2,7'-cycloligna-7,7'-dien-9,9'-olide, 45 mg), savinin (3,4:3',4'-bis(methylenedioxy)-lign-7-en-9,9'-olide, 33 mg), taiwanin C (3',4':4,5-bis(methylenedioxy)-2,7'-cycloligna-7,7'-dien-9,9'-olide, 27 mg), and *cis*-dibenzylbutyrolactone (3-(3,4-dimethoxybenzyl)-2-(3,4-methylenedioxybenzyl)butyrolactone, 45 mg). The five lignans isolated from the roots of *Acanthopanax chiisanensis* (Fig. 12) were identified by a comparison of spectral data (NMR, IR, UV) with authentic compounds.

Preparation and culture of rat peritoneal macrophages

A solution of soluble starch (Wako Pure Chemicals, Osaka, Japan) and Bactopeptone (Difco, Detroit, MI, USA), 5% each, was injected intraperitoneally into male Sprague-Dawley rats (400-550 g, specific pathogen-free, Charles River Japan, Kanagawa, Japan) at a dose of 5 ml per 100 g body weight. Four days later, the rats were killed by cutting the carotid artery under anesthesia, and the peritoneal cells were harvested [113]. The experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals approved

by the Animal Ethics Committee of the Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan. The peritoneal cells suspended in Eagle's minimal essential medium (EMEM, Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% calf serum (Flow Laboratories, North Rydge, Australia), penicillin G potassium (18 μg/ml) and streptomycin sulfate (50 μg/ml) (Meiji Seika, Tokyo, Japan) were seeded at a density of 7.5 x 10^5 cells/0.5 ml/well in 24-well plastic tissue culture dishes (Corning Glass Works, Corning, NY, USA) or 3.0 x 10^6 cells/2 ml/well in 6-well plastic tissue culture dishes (Corning), and incubated for 2 h at 37°C under 5% CO_2-95% air. The wells were then washed three times with medium to remove non-adherent cells, and the adherent cells were further incubated at 37°C for 20 h. After three washes with PBS, the adherent cells were used for subsequent experiments.

Culture of RAW 264.7 cells

RAW 264.7 cells were obtained from RIKEN Gene Bank and incubated at 37°C under 5% CO_2-95% air in EMEM supplemented with 10% fetal bovine serum (FBS, Sigma Chemical, St Louis, MO, USA). For subsequent experiments, the cells were seeded at a density of 5 x 10^5 cells/0.5 ml/well in 24-well plastic tissue culture dishes or 2 x 10^6 cells/2 ml/well in 6-well tissue culture dishes and incubated for 20 h at 37°C.

Culture of RBL-2H3 cells

RBL-2H3 cells were obtained from Health Science Research Resources Bank and incubated at 37°C under 5% CO_2-95% air in EMEM supplemented with 10% FBS and 0.5% conditioned medium of DNP-specific IgE-producing hybridoma kindly supplied by Dr. Kazutaka Maeyama (Ehime University, Ehime, Japan). For subsequent experiments, the cells were seeded at a density of 2.5 x 10^5 cells/0.5 ml/well in 24-well plastic tissue culture dishes, 1 x 10^6 cells/2 ml/well in 6-well tissue culture dishes, or 5 x 10^6 cells/10 ml/dish in p100 cell culture dishes (Corning) and incubated for 20 h at 37°C.

Drugs used

The drugs used were LPS (Wako), the NF-κB inhibitor Bay II-7082 (Calbiochem,

Darmstadt, Germany), the MEK inhibitor U0126 (Calbiochem), the p38 MAPK inhibitor SB203580 (Calbiochem), the PI3K inhibitor wortmannin (Plymouth Meeting, Pa, USA), the JNK inhibitor SP600125 (Plymouth), the MEK inhibitor PD98059 (New England Biolabs, Beverly, MA, USA), the tyrosine kinase inhibitor genistein (Sigma), the protein kinase C activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA, Sigma), the endomembrane Ca^{2+}-ATPase inhibitor thapsigargin (Wako), the non-specific protein kinase inhibitor staurosporine (Kyowa Medex, Tokyo, Japan), the synthetic glucocorticoid dexamethasone (Sigma), the COX-1/COX-2 non-specific inhibitor indomethacin (Wako) and aspirin (Sigma), the COX-2 specific inhibitor NS-398 (Calbiochem), arachidonic acid (Sigma), DNP-HSA (sigma), the Syk inhibitor piceatannol (Sigma), and resveratrol (Wako). Each drug was dissolved in DMSO or ethanol, and added to the medium. The final concentrations of DMSO and ethanol in the medium were adjusted to 0.1% (v/v), respectively. The control medium contained the same amount of DMSO and ethanol.

Measurement of PGE_2, TNF-α, IL-13 and LTC_4 concentration

After incubation for the specified period, the conditioned medium was collected, and centrifuged for 5 min at 1,500 x *g* and 4°C. The concentration of PGE_2 in the supernatant was determined by radioimmunoassay [113]. Anti-PGE_2 antibody was purchased from Assay Designs (Ann Arbor, MI, USA). The concentrations of TNF-α, IL-13 and LTC_4 in the supernatant were determined by TNF-α ELISA kit (BioSource International, Camarillo, CA, USA), IL-13 ELISA kit (BioSource) and LTC_4 EIA kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions, respectively.

COX-1 and COX-2-dependent PGE_2 production

The rat peritoneal macrophages (1.5 x 10^6 cells/ml) were preincubated for 4 h at 37°C in 0.5 ml of medium containing dexamethasone (10 μM). After three washes, the adherent cells were further incubated for 4 h at 37°C in 0.5 ml of medium containing dexamethasone (10 μM) and each drug in the presence of arachidonic acid (10 μM). After the incubation, the concentration of PGE_2 in the conditioned medium was radioimmunoassayed (COX-1-dependent PGE_2 production). Another set of macrophages (7.5 x 10^5 cells) was preincubated

for 4 h at 37°C in 0.5 ml of medium containing aspirin (100 μM). After three washes, the adherent cells were further incubated for 4 h at 37°C in 0.5 ml of medium containing each drug in the presence of TPA (30 nM). After the incubation, the concentration of PGE_2 in the conditioned medium was radioimmunoassayed (COX-2-dependent PGE_2 production) [114].

Determination of activities of COX-1 and COX-2 in a cell-free system

Activities of COX-1 and COX-2 in a cell-free system were determined according to the method described by Mancini *et al.* [115] and Kim *et al.* [116]. One unit of COX-1 (isolated from sheep seminal vesicles, purity 95%, Cayman) or COX-2 (isolated from sheep placenta, purity 70%, Cayman) was dissolved in 210 μl of Tris-HCl (100 mM, pH 7.4) containing 10 mM EDTA, 1 mM reduced glutathione, 1 μM hematin and 0.5 mM phenol. The reaction mixture was preincubated for 3 min at 37°C with various concentrations of indomethacin, NS-398, or taiwanin C. After addition of arachidonic acid (0.1 μM), the mixture was further incubated for 3 min at 37°C. To terminate the reaction, 20 μl of 1 M HCl was added to the reaction mixture. An equivalent volume of 1 M NaOH was then added to neutralize the mixture, and the amount of PGE_2 was measured by radioimmunoassay.

Measurement of radioactivity released from [^{3}H]arachidonic acid-labeled cells

The rat peritoneal macrophages (1.5 x 10^6 cells) or RBL-2H3 cells (5 x 10^5 cells) were incubated for 20 h at 37°C in 0.5 ml of medium containing 3.7 kBq of [^{3}H]arachidonic acid (2.26 TBq/mmol, PerkinElmer Life Sciences, Boston, MA, USA). The cells were washed three times with medium to remove free [^{3}H]arachidonic acid, and incubated for the periods indicated at 37°C in 0.5 ml of medium containing each drug. The conditioned medium was withdrawn at 1, 2 or 4 h, centrifuged for 5 min at 1,500 x *g* and 4°C, and the radioactivity in the supernatant were determined [117].

Immunoblotting

After incubation for the specific period, the cells were washed three times with PBS, dipped in 150 μl of ice-cold lysis buffer (20 mM HEPES, 1% Triton-X 100, 10% glycerol, 1 M sodium fluoride, 2.5 mM *p*-nitrophenylene phosphate, 10 μg/ml of phenylmethylsulfonyl-

fluoride, 1 mM sodium vanadate, 5 μg/ml of leupeptin, and 1 mM EDTA, pH 7.4) for 15 min, and centrifuged for 20 min at 13,000 x *g* and 4°C. The supernatant was boiled for 5 min in 3 x sample buffer (50 mM Tris, 4% SDS, 10% glycerol, 4% 2-mercaptoethanol, and 0.05 mg/ml of bromophenol blue, pH 7.4) at a ratio of 2:1 (v/v), loaded on an acrylamide gel (8 to 16%) and subjected to electrophoresis (120 min at 125 V). The separated proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) at 150 mA for 1 h. After transfer onto the nitrocellulose membrane, the blot was incubated in blocking solution (Block Ace, Dainippon, Osaka, Japan) at room temperature for 1 h and incubated overnight at 4°C with primary antibody. The blot was then incubated for 3 h at 4°C with secondary antibody anti-goat IgG (Vector Laboratories, Burlingame, CA) or anti-rabbit IgG (Vector). Finally, it was incubated with Vectastain ABC reagent (Vector) at room temperature for 30 min, then with ECL reagent (Amersham International, Amersham, UK) at room temperature for 1 min, and exposed to Kodak Scientific Imaging Film. Antibodies used were anti-COX-1 (Santa Cruz Biotechnology, CA, USA), anti-COX-2 (Santa Cruz), anti-cPGES (Cayman), anti-mPGES-1 (Cayman), anti-IκB-α (Santa Cruz), anti-iNOS (Santa Cruz), anti-actin (Santa Cruz), anti-phospho-Akt (Ser473, Cell Signaling, Beverly, MA, USA), anti-Akt (Cell Signaling), anti-phospho-c-Jun (Ser73, Cell Signaling), anti-c-Jun (H-79, Santa Cruz), anti-phospho-p44/42 MAP kinase (Thr202/Tyr204, Cell Signaling), anti-MAP kinase R2 (Rek1-CT, Upstate Biotechnology, Lake Placid, NY, USA), anti-phospho-p38 MAPK (Thr180/182, Cell Signaling), anti-p38 MAPK (Santa Cruz), anti-Syk (N-19, Santa Cruz), anti-phospho-Pyk2 antibody (Tyr402, Santa Cruz), anti-Pyk2 (H-102, Santa Cruz), and anti-phosphotyrosine (4G10, Upstate).

Reverse transcription (RT)-polymerase chain reaction (PCR)

After incubation for the specific period, the total RNA was extracted using a VIOGENE DNA/RNA Extraction kit (Viogene, Sunyvale, CA, USA) according to the manufacturer's instructions. The extracted RNA (1 μg) was reverse transcribed for 1 h at 37°C by adding 5 μM of random hexamer oligonucleotides (Gibco BRL, Gaithersburg, MD, USA), 200 units of reverse transcriptase (Takara Bio, Shiga, Japan), 0.5 mM deoxyribonucleotide triphosphates (dNTP) (Takara) and 10 mM dithiothreitol (Takara). The PCR primers used

were 5'-TTG ACC TCA GCG CTG AGT TG-3' (sense) and 5'-CCT GTA GCC CAC GTC GTA GC-3' (antisense) for TNF-α, 5'-TCG CTT GCC TTG GTG GTC TT-3' (sense) and 5'-CTG GGC TAC TTC GAT TTT GG-3' (antisense) for IL-13, and 5'-TGA TGA CAT CAA GAA GGT GGT GGA-3' (sense) and 5'-TCC TTG GAG GCC ATG TAG GCC AT-3' (antisense) for GAPDH. PCR for TNF-α was performed for 27 cycles at 94°C for 30 s, at 58°C for 1 min and at 72°C for 1.5 min using a DNA thermal cycler (Takara), for IL-13, with 30 cycles at 94°C for 30 s, at 60°C for 30 s and at 72°C for 45 s, and for GAPDH, with 27 cycles at 94°C for 1 min, at 57°C for 1 min and at 72°C for 1 min. PCR was carried out with 10 μl of template DNA and 40 μl of PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 1.5 mM $MgCl_2$) containing each primer (0.2 μM), dNTP (0.2 mM) and *Taq* DNA polymerase (1.25 units) (Takara). After PCR, 10 μl of the reaction mixture was subjected to electrophoresis on a 1.5% agarose gel, and the PCR products were visualized by ethidium bromide staining. The levels of mRNA for TNF-α, IL-13 and GAPDH were quantified by scanning densitometry.

Preparation of nuclear extract

After incubation for the specific period, the cells were scrapped off the plate using a cell scraper, and centrifuged for 5 min at 2,500 x *g* and 4°C. The cells were suspended in 400 μl of Tris-buffered KCl solution (20 mM Tris-HCl, 50 mM KCl, 10 μg/ml of leupeptin, 0.1 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride, pH 7.8), and lysed by the addition of the same volume of Tris-buffered KCl solution containing 1.2% Nonidet P40 with vigorous mixing for 10 s. The homogenate was centrifuged for 30 s at 4°C and 15,000 x *g*, and the nuclear pellet was suspended in 30 μl of cold Tris-buffered KCl solution by mixing for 15 min at 4°C. The suspension was then centrifuged for 20 min 15,000 x *g* and at 4°C, and the resultant supernatant (nuclear extract fraction) was stored at -80°C prior to use.

Electrophoretic mobility shift assay (EMSA)

EMSA was carried out according to the protocol accompanying the Gel Shift Assay System (Promega, Madison, WI, USA). Briefly, the double-stranded oligonucleotide probes

containing NF-κB- and AP-1-binding sequences (Promega) were end-labeled with 1.85 MBq of [γ-^{32}P] ATP (111 TBq/nmol, PerkinElmer Life Sciences) using T4 polynucleotide kinase. The nuclear extract (4 μg) was incubated at room temperature for 20 min with 4 μl of [γ-^{32}P]-labeled probe in a binding buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 0.25 mg/ml of poly (dI-dC), and 20% glycerol, pH 7.5). DNA/nuclear protein complexes were separated from the DNA probe by electrophoresis on a native 4% acrylamide gel, and the gel was vacuum-dried and visualized with a GS-250 Molecular Imager (Bio-Rad).

Luciferase assay

RAW 264.7 cells were suspended in K-PBS (30.1 mM NaCl, 120.1 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4), and their concentration was adjusted to be 1.5 x 10^7 cells/ml with K-PBS. From the cell suspension, 400 μl (6 x 10^6 cells) was transferred into a 15 ml tube, and 30 μg of pNF-κB-Luc (Stratagene, La Jalla, CA, USA) was added. After standing at room temperature for 10 min, the cell suspension containing pNF-κB-Luc was transferred into a 2 mm Gap Cuvette, (BTX Technologies, Hawthorne, NY USA) and performed an electroporation using a Electro Square Porator ECM 830 (BTX) (Mode: LV, Voltage: 150 V, P. length: 15 ms, Pulse: 1). The transfected cells (6 x 10^6 cells/430 μl) were suspended in 11.57 ml of EMEM containing 10 % FBS, and 0.5 ml of the cell suspension was poured in each well of a 24-well culture plate and incubated for 12 h at 37°C under 5% CO_2-95% air. After 6 h incubation, the cells were washed three times with PBS, and dipped in 50 μl of the lysis buffer for 15 min at room temperature. The lysate was transferred into a 1.7 ml micro centrifuge tube, and centrifuged for 2 min at 13,200 x *g*. The supernatant fraction was used as a sample for luciferase assay. For luciferase assay, 20 μl of the sample was transferred into a luciferase assay tube, and 20 μl of the assay buffer (0.2 M Tricine-NaOH, 0.1 M $(MgCO_3)_4Mg(OH)_2$, 1 M $MgSO_4$, 0.5 M EDTA, 0.1 M DTT, 2.7 mM CoA, 47 mM luciferine, and 0.1 mM ATP, pH 7.8) was added into the tube containing the sample. The luciferase activity was measured using a Luminescencer-PSN (AB-2200, ATTO, Tokyo, Japan).

Degranulation of RBL-2H3 cells

The degree of the antigen-induced degranulation of RBL-2H3 cells was assessed by measuring the percentage of hexosaminidase released [118]. After stimulation with the antigen for 15 min, 20 μl of the conditioned medium was withdrawn and mixed with 20 μl of 0.1 M citrate buffer (pH 4.5) containing 1 mM *p*-nitrophenyl *N*-acetyl β-D-glucosamide (Sigma). The solution was incubated for 1 h at 37°C. The reaction was stopped by the addition of 100 μl of 0.1 M sodium carbonate buffer (pH 10.0) and the absorbance at 405 nm was measured. Before the antigen stimulation, the cells in one set of groups were lysed with 0.1% Triton X-100 (Wako) to determine the total amount of hexosaminidase in the cells. The percentage of the hexosaminidase released was then calculated.

Immunoprecipitation

RBL-2H3 cells (5 x 10^6 cells) were dipped in 500 μl of ice-cold RIPA buffer (PBS, 1% (w/v) Nonidet P40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1M sodium fluoride, 2.5 mM *p*-nitrophenylene phosphate, 10 μg/ml of phenylmethylsulfonylfluoride, 1 mM sodium vanadate, and 5 μg/ml of leupeptin) for 30 min at 4°C. The RIPA buffer containing the cells was centrifuged for 20 min at 13,200 x *g* and 4°C, and the supernatant obtained was incubated overnight at 4°C with 20 μl of anti-Syk antibody agarose conjugate (N-19AC, Santa Cruz).

Statistical analysis

The statistical significance of the results was analyzed by Dunnett's test for multiple comparisons and Student's *t*-test for unpaired observations.

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List of Publications Related to This Thesis

1. **Inhibition of prostaglandin E_2 production by taiwanin C isolated from the root of *Acanthopanax chiisanensis* and the mechanism of action**
   Ban HS, Lee S, Kim YP, Yamaki K, Shin KH, Ohuchi K
   Biochemical Pharmacology 64:1345-1354 (2002)

2. **Inhibitory effects of furanocoumarins isolated from the roots of *Angelica dahurica* on prostaglandin E_2 production**
   Ban HS, Lim SS, Suzuki K, Jung SH, Lee S, Lee YS, Shin KH, Ohuchi K
   Planta Medica 69:408-412 (2003)

3. **Inhibition of lipopolysaccharide-induced expression of inducible nitric oxide synthase and tumor necrosis factor-α by 2'-hydroxychalcone derivatives in RAW 264.7 cells**
   Ban HS, Suzuki K, Lim SS, Jung SH, Lee S, Ji J, Lee HS, Lee YS, Shin KH, Ohuchi K
   Biochemical Pharmacology 67:1549-1557 (2004)

4. **Lignans from *Acanthopanax chiisanensis* having an inhibitory activity on prostaglandin E_2 production**
   Lee S, Ban HS, Kim YP, Kim BK, Cho SH, Ohuchi K, Shin KH
   Phytotherapy Research 19:103-106 (2005)

5. **Inhibition by resveratrol (*trans*-3,4',5-trihydroxystilbene) of FcεRI-mediated activation of RBL-2H3 cells**
   Ban HS, Hirata Y, Aoyama S, Ohuchi K
   European Journal of Pharmacology manuscript in preparation (2006)